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THE PROLONGED ADMINISTRATION OF THYROXINE TO COWS, WITH PARTICULAR REFERENCE TO THE EFFECTS ON THYROID FUNCTION AND ON PITUITARY THYROTROPHIC HORMONE.

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DURING recent years several papers have appeared recording the results of the administration of thyroxine or thyroxine-containing proteins to the cow, pertaining chiefly to the influence of such treatment upon milk production during the middle and later periods of lactation (Reineke and Turner, 1942 ; Blaxter, 1945, *a*, *b* ; 1946). The main purpose of the present investigation was to study in this animal the effect of thyroxine administration upon the functions of the thyroid and pituitary glands.

For the laboratory animal, thyroxine has been shown to depress the activity of the thyroid gland (Belasco and Murlin, 1941 ; Cortell and Rawson, 1944), and to reduce the stimulating influence on this gland of thyrotrophic hormone concurrently administered (Aron, 1930 ; Salter, 1940). Hohlweg and Junkmann (1933) and Kuschinsky (1933), in the rat, and Adams and Jensen (1944), in the mouse, found that continued treatment with thyroxine led to a marked diminution in the thyrotrophic hormone content of the pituitary gland. Reforzo-Membrives (1943) further made the important claim that the prolonged (22 days) feeding of relatively large amounts of thyroid powder to rats actually brought about a reversal of pituitary function, with the development of a thyroid-inhibitory substance by this gland. This finding could not, however, be substantiated by Purves and Griesbach (1946).

From the larger animals at our disposal, we have obtained data on the changes in the iodine status of the thyroid gland and in the thyrotrophic potency of the

anterior lobe of the pituitary gland induced by injections of thyroxine over ten weeks. Clinical findings were recorded, and the opportunity was also taken to ascertain whether cellular changes had occurred in various tissues as the result of this treatment. In addition, certain observations were made on the blood to provide data supplementing those obtained by one of us (A. W. T.) in connection with studies on the effects of loss of condition on the immune state.

MATERIALS AND METHODS.

The experimental animals were 15 Shorthorn cows, aged about 2½ years, which had been previously used in an experiment concerned with brucellosis, for which purpose they had been mated when about 20 months old. Numbers C12, C16, T21, T25, T26, T31, T33 had aborted between the fifth and the seventh month of pregnancy as a result of infection with virulent *Brucella abortus* and had, therefore, never suckled; the remaining 8 animals, having been vaccinated intracaudally with living *Br. abortus* "Strain 19" about 3 months before being mated, had successfully withstood exposure to infection by the virulent organism, had calved normally at full term and had experienced normal lactation, which was terminated at 79 to 142 days before the beginning of the experiment. Parturition or abortion had occurred from 3–6 months before the animals were taken into the present experiment; at this stage they were neither pregnant nor lactating and were all in good condition, being classed as "forward stores." They were confined to a paddock of 28 acres with an excellent pasture consisting mainly of *Phalaris tuberosa*, *Lolium multiflorum* and *Trifolium subterraneum* (Mt. Barker mid-season strain), and were thus provided with abundant high class nutriment *ad libitum*. The experiment was carried out between the middle of March and the middle of June, 1946, i.e. the autumn months, during which the mean monthly maximal dry-bulb shade temperature fell fairly steadily from 71° F. to 55° F., and the mean monthly minimal dry-bulb shade temperature from 54·6° F. to 44·4° F. Frequent light rains were recorded throughout the period, the totals for March, April, May and June being 3·52, 2·33, 1·26 and 3·12 inches respectively, which ensured a steady growth of pasture.

The animals were first divided into 12 ranging in weight from 567 to 796 lb., and 3 larger animals ranging from 821 to 900 lb. The main group was subdivided into 6 pairs as evenly matched in weight as possible, the differences between members of pairs being from 4 to 54 lb. The members of the matched pairs were distributed randomly to either of 2 groups, which were then allotted randomly as "controls" (Group I: No. C11–C16), or "treated" (Group II: No. T21–T26). The 3 larger unmatched animals formed a separate sub-group of treated animals (Group III: No. T31–T33), which, in the statistical analysis, was usually combined with the main treated group, since it was found that it did not differ significantly from this group, but, in the preparation of the graphs, the data from only the 12 matched animals have been used.

The experiment comprised 4 periods, beginning with a training period (Period 1) of 16 days, during which the animals were accustomed to the routine of handling and clinical examination. Although precise information as to an appropriate dosage of thyroxine was not available, we were guided by the experience of Folley and White (1936) and of Blaxter (1945*a*), who showed that 10 mg. per day had a significant effect on milk production. The initial dosage chosen was 15

mg., and this was increased as it seemed desirable ; during Period 2 (15 days), Period 3 (25 days) and Period 4 (30 days) the treated animals accordingly received subcutaneously thrice weekly 15, 20 and 40 mg. respectively of thyroxine, dissolved in physiological saline by warming with a little 0·05 N NaOH. The total quantity of thyroxine administered to each animal during the 70 days of treatment was 805 mg., the amounts for the three periods being 105 mg., 220 mg. and 480 mg. respectively. A British Drug Houses B.P. specimen and one resulting from the iodination of 3:5-di-iodo-thyronine by the method of Niemann and Mead (1941) provided the thyroxine used. The di-iodo-thyronine had been donated by the firm of Schering, A. G. The observations and treatments were carried out during mornings, always at approximately the same hour, the animals being moved as quietly as possible in batches into a crush for the taking of respirations, heart beats (by means of a stethoscope), and rectal temperatures. Once weekly all were weighed (after an overnight period without water) and, every second week, 5 ml. of jugular blood was removed by syringe and transferred to a tube containing 0·1 ml. of a solution of heparin containing 1000 units of heparin per ml. The blood samples were examined at the laboratory ; a sample was centrifuged, and the specific gravity of whole blood and of plasma was determined by the copper-sulphate method of Phillips, Van Slyke, Dole, Emerson, Hamilton and Archibald (1945).

At the beginning and at the end of the experiment agglutination tests against *Br. abortus* were carried out with a series of doubling dilutions, starting from 1:10 dilution of serum. The titres are recorded in the $\log_2$ titre/10 notation as described by Campbell and Rodwell (1945).

Post-mortem procedures.

Specimens.—In the "treated" groups, treatment ceased on June 8, 1946, observations ceased on June 11, and autopsies were carried out at abattoirs on June 12, i.e. 4 days after the last injection ; in the " control " group, final observations were made on June 18 and autopsies on June 19.

At autopsy the following materials were fixed in buffered 10 per cent formalin saline : adrenal and ovarian glands and portions of mammary and thyroid glands, liver, kidney, uterus and spinous process of the 1st thoracic vertebrae. The pituitary glands from 3 treated animals (T26 (Group II), T32 and T33 (Group III)), and, subsequently, those from a range of normal animals obtained at abattoirs were added to mercuric chloride fixative. The bony tissue was decalcified with 10 per cent nitric acid in 5 per cent formalin solution. All tissues were embedded in paraffin, and the sections were stained with haematoxylin plus eosin or Van Gieson stain, those of the pituitaries by Mallory's trichrome method.

Thyroid and pituitary assays.—Acinar cell heights of the thyroid glands were estimated by measuring 50 acini per section, using an etched scale on thin glass, with an opaque screen, at a magnification of 1000 (Griesbach and Purves, 1943).

One thyroid gland (cleared of extraneous tissue) from each cow was sliced, dried for 24 hours at 40° C. (to constant weight), and the thyroid powder then extracted with ether for 40 hours. The iodine content was determined according to Baumann and Metzger's (1937) modification of Leipert's method. For the estimation of " thyroxine iodine," hydrolysis of the thyroid powder (100 mg.)

and extraction of " thyroxine " was carried out according to the method of Reineke and Turner (1945) and iodine determined as above. All samples were estimated in duplicate.

Pituitary glands from 6 treated animals—5 of the 6 animals from Group II (T21–T25) and one from Group III (T31)—and from all control animals (C11–C16) were wrapped in parafilm and transferred to a dry ice container. While still frozen, anterior lobes were freed from the median and posterior lobes ; they were then dehydrated with acetone at 0°, washed with peroxide-free ether and finally dried over P_2O_5 *in vacuo* and powdered. Prior to assay, the anterior pituitary powder (A.P.P.) from the control group was pooled, as also was that from the five glands obtained from the animals of Group II. The pituitary gland from animal T31 was kept separate and used only for a preliminary experiment. The two-day-old White Leghorn cockerel was used for the determination of thyrotrophic potency, the appropriate amount of A.P.P. being ground with saline just prior to injection. Comparisons were also made with a highly potent standardized preparation of bovine thyrotrophic hormone, donated by Schering, A. G., and containing 34 G.P. units per mg. (Junkmann and Schoeller, 1932). Injections were given intraperitoneally for four days, control chicks receiving saline. In the case of two of the chick groups, one serving as an additional control group and the other receiving A.P.P. from thyroxine-treated cows, the injections were given for 10 days in order to provide a comparison at a higher acinar-cell-height level. At the conclusion of the experimental period thyroids were dissected and prepared for histological examination (eosin-haematoxylin stain). Acinar cell heights were measured as an estimate of thyroid activity, using the technique mentioned above.

Statistical treatment of results.—The several sets of data have been treated by means of the analysis of variance. For the quantitative clinical findings the trend of the difference in any characteristic, between treated and control cows, rather than the actual differences, was studied. The significance of the various effects has been indicated in the summarized analysis of variance table (Table I). Thus, the significance of the trend with time of the difference between the treated and control groups is shown as period-treatment interaction. For the thyroid and pituitary assays, where direct comparisons of means are relevant, the means and standard errors have been tabulated and the significant differences indicated (Tables III and IV).

RESULTS.

Clinical findings.

General.—After about 3 weeks from the commencement of thyroxine administration the treated animals began to become nervous and excitable ; they developed a wild, " staring " appearance, were restless, and readily defaecated and urinated during handling. At rest their respirations appeared faster than those of the controls. Their udders began to develop during the course of the treatment, so that they eventually resembled those normally seen at the 6th or 7th month of pregnancy ; the teats became turgid, and up to 30 ml. of milky secretion could be readily expressed, whereas in the control group only a small quantity of slightly yellowish-brown watery fluid could be obtained. The treated animals failed to " fatten " as quickly as the controls and were noticeably " poor " and

"unthrifty." Oestrus was rarely observable among either the control or treated animals.

Body weight.—Failure to "fatten" was borne out by the weight records (Fig. 1). At the beginning of the experiment the mean weights of the 6 "controls" was 673·7 lb. ± 28·1 (S.E.M.), and that of the 6 matched "treated" animals was 680·7 lb. ± 32·8 lb. The controls steadily gained weight at a satisfactory rate, so that at the 12th weighing, when observations ceased, the mean weight was 846·5 lb. ± 28·7 lb., corresponding to a mean gain of 172·8 lb.; on the other hand, the matched treated animals slowly reached only 733 lb. ± 33·0 lb., corresponding to a mean gain of 52·3 lb. In the sub-group of the three larger treated animals the mean weight increased from 854·7 lb. to 870·0 lb., with a mean gain of 15·3 lb.

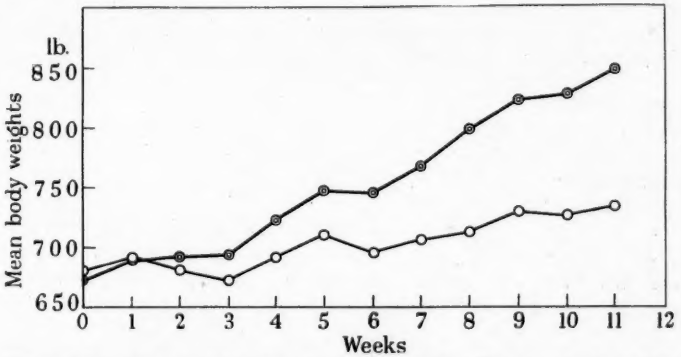


FIG. 1.—Effect of thyroxine administration upon body weight. The mean body weights of the two matched groups are plotted at weekly intervals. (Full circles = controls; open circles = treated.)

TABLE I.—*Clinical Data from Control and Thyroxine-treated Cows (Combined Treated Groups II and III, i.e. 9 Animals). Summarized Analysis of Variance Tables.*

	Degrees of freedom.	Sum of squares.	Mean square.
Body weight.			
Treatment and periods	3	68,744	22,910**
Cows and periods	36	21,926	609·1
Respiratory frequency.			
Treatments and periods	3	25,607	8,536**
Cows and periods	36	32,112	892·0
Cardiac frequency.			
Treatments and periods	3	23,160	7,720**
Cows and periods	36	16,360	454·4
S.G. of whole blood.			
Treatments and samples	5	8,192	1,638**
Cows and samples	58	19,865	342·5

The significance of each effect is indicated against the corresponding mean square, thus: ** = significant at 1 per cent level.

In the statistical analysis (Table I) the matched and unmatched treated animals were combined and their mean weights compared with those of the controls. There was a significant variation in the differences between the treated and the controls for the different observations within any of the four periods. The differences between treated and control animals show a trend significant at the 1 per cent level.

Respiratory frequencies.—These varied greatly even in the same animals from day to day both in control and treated animals, possibly the result of exertion and excitement associated with being passed through the crush for examination, accentuated by the restlessness of the more excitable treated animals. However, it was evident that in spite of this the trends were very definite, and this was borne out when the fluctuations were smoothed out by determining the mean frequencies for the groups during each of the four experimental periods. As shown in Fig. 2, in which these values as ordinates are plotted against the mid-points of each period as abscissae, the mean frequencies of the controls, after a

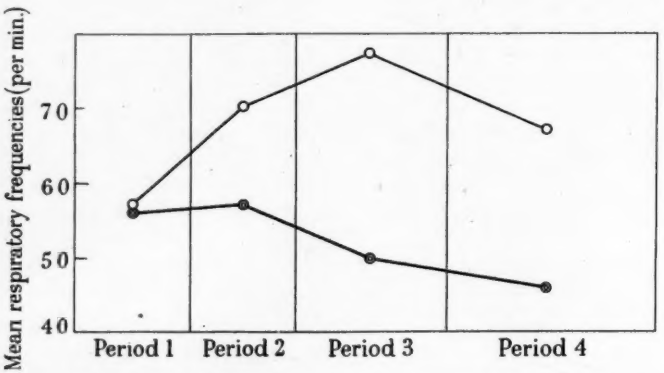


FIG. 2.—Effect of thyroxine administration upon respiratory frequency. The mean frequencies of the 2 matched groups for each of the 4 experimental periods are plotted as ordinates against the mid-point of the periods as abscissae. (Full circles = controls; open circles = treated.)

slight rise, gradually fell from about 56 per minute to about 46, whereas those of the matched treated animals rose to about 70 as a result of receiving 15 mg. thyroxine thrice weekly, reached a maximum of about 77 when receiving 20 mg. thyroxine, and fell to 68 during the final period when receiving 40 mg. thyroxine.

Since there was no significant over-all difference between the 6 matched treated and the 3 large treated animals, they were combined for the statistical analysis (Table I), which showed that the mean respiratory frequency increased significantly, at the 1 per cent level, for the treated animals, in comparison with the controls, during the combined periods of treatment.

Cardiac frequencies.—Here again the individual daily fluctuations were very great, but, by adopting the same method of smoothing out by grouping the data, a very similar sequence of events is demonstrated (Fig. 3); mean group frequencies of the controls during the experimental periods fell steadily from about 75 to about 69, after a preliminary slight rise, whereas those of the animals in the

combined treated groups rose sharply to a maximum of 97, which was maintained during Periods 3 and 4. Statistical analysis (Table I) showed that the increase was significant, at the 1 per cent level, over the three periods of treatment.

Rectal temperatures.—Fluctuations were rather large, but grouping of the data as before smoothed these out. The mean group rectal temperatures during each of the four experimental periods are set out in Table II, which demonstrates that the differences are not significant.

TABLE II.—*Effect of Thyroxine Administration upon Rectal Temperatures.*

Periods.	Pre-treatment.	Treatment.		
	1.	2.	3.	4.
Control	102·5	102·4	102·0	102·1
Treated	102·3	102·4	102·2	102·2
Difference	−0·2	0	+0·2	+0·1

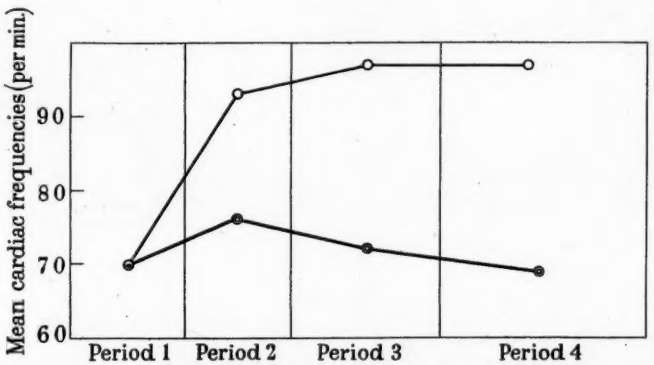


FIG. 3.—Effect of thyroxine administration upon cardiac frequency. The mean frequencies of the 2 matched groups for each of the 4 experimental periods are plotted as ordinates against the mid-point of the periods as abscissae. (Full circles = controls; open circles = treated.)

Specific gravity (S.G.) of blood and of plasma.—During the course of the experiment the mean S.G. of blood in the controls increased from 1·0517 at the first examination to 1·0556 at the fifth, falling at the final (sixth) examination to 1·0537. As shown in Fig. 4, the general trend was thus a considerable increase in the S.G. of whole blood which did not occur with plasma. In the treated group there was no marked increase in the S.G. of blood which, from the third examination onwards, closely paralleled the fluctuations in the control group, but at a lower level, so that from an initial value of 1·0525 it actually fell at the final examination to 1·0508; meanwhile the plasma S.G. values fell from an initial value of 1·0288 to a final of 1·0269. The meaning of the fluctuations, particularly the sharp rise of S.G. of blood in both groups at the fifth examination, is not clear, but is probably related to environmental variations. For the S.G. of blood the difference in trend was significant at the 1 per cent level, whereas for plasma it was not.

The inter-relationships between the S.G. of blood and plasma on the one hand and of plasma-protein concentration and red-cell count on the other hand have not been formulated yet for bovines, and therefore inferences based upon the nomogram of Phillips *et al.* (1935) for human blood can have only relative value. With this reservation, it appears that during the course of the experiment the red cell count of the controls in contrast to that of the treated animals increased without an increase in plasma proteins.

Antibody content of sera.—As a result of vaccination or infection with *Br. abortus*, all animals had at the beginning of the experiment agglutinin titres with the usual wide individual variations. Examinations at the end of the experiment showed that the changes neither within groups nor between groups were significant, and that there was no significant difference between the extent of the change in titres in the treated as compared with the control animals; in the

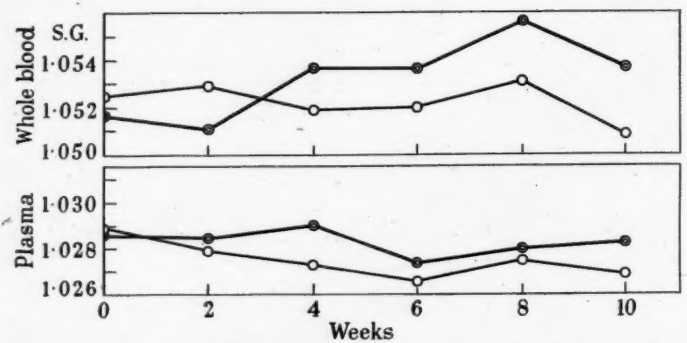


FIG. 4.—Effect of thyroxine administration upon specific gravity of blood and of plasma. The mean S.G. of the 2 matched groups are plotted at fortnightly intervals. (Full circles = controls; open circles = treated.)

treated groups the logarithms to the base 2 of the geometric mean titres fell from $5{\cdot}8 \pm 0{\cdot}50$ to $4{\cdot}8 \pm 0{\cdot}40$, whereas in the controls they rose from $5{\cdot}00 \pm 0{\cdot}42$ to $5{\cdot}7 \pm 0{\cdot}39$.

EXPLANATION OF PLATES.

FIG. 5 and 7.—Effect of thyroxine administration upon mammary glands. Control Cow C14, showing poorly developed duct system and much adipose tissue. (×14 and × 49 resp.)

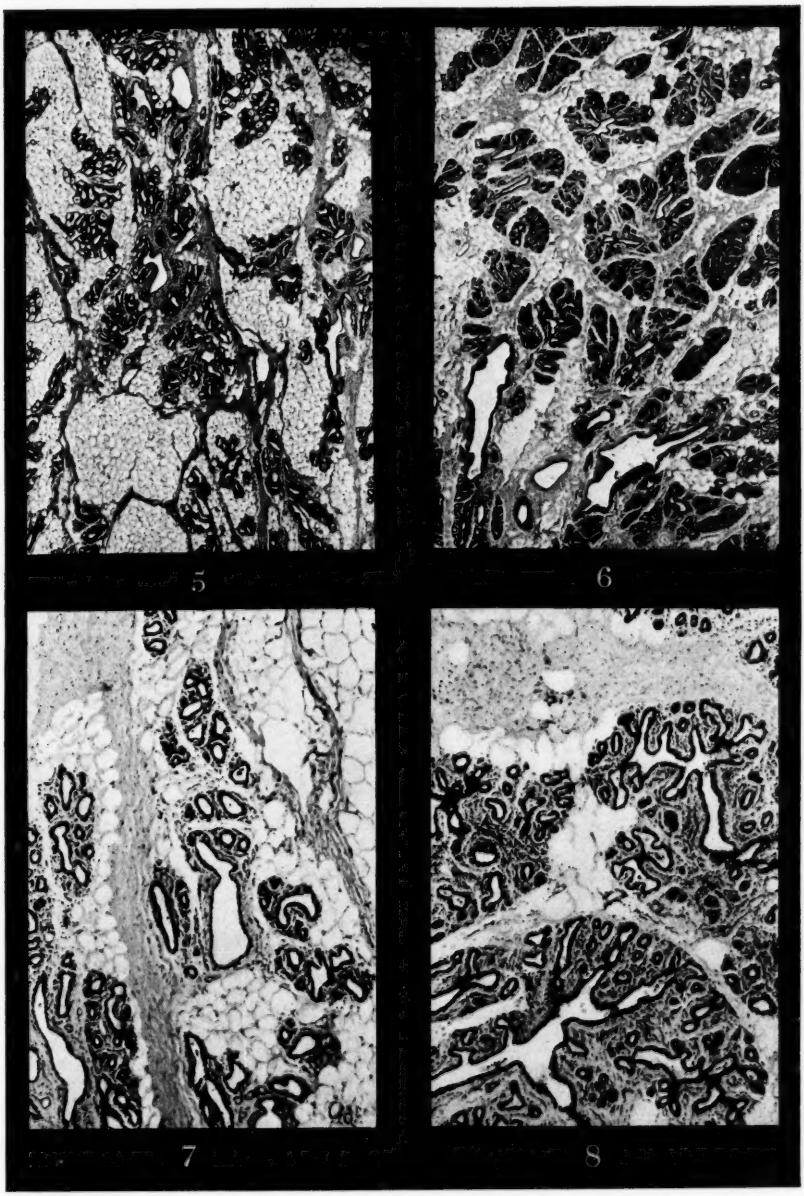
FIG. 6 and 8.—Effect of thyroxine administration upon mammary glands. Treated Cow T26 showing greater lobule development associated with increased duct proliferation and less adipose tissue. (× 14 and × 49 resp.)

FIG. 9 and 11.—Effect of thyroxine administration upon thyroid glands. Control Cow C12, showing plump cuboidal acinar cells and dark-staining colloid. (× 14 and × 340 resp.)

FIG. 10 and 12.—Effect of thyroxine administration upon thyroid glands. Treated Cow T23, showing low flattened acinar cells and lighter-staining acinar contents. (× 14 and × 340 resp.)

FIG. 13 and 15.—Effect of thyroxine administration upon spinous process of 1st thoracic vertebrae. Control Cow C12, showing cartilage and bone. (× 4 and × 24 resp.)

FIG. 14 and 16.—Effect of thyroxine administration upon spinous process of 1st thoracic vertebrae. Treated Cow T24, showing nutritive canals in cartilage but no obvious difference in bony trabeculae. (× 4 and × 24 resp.)



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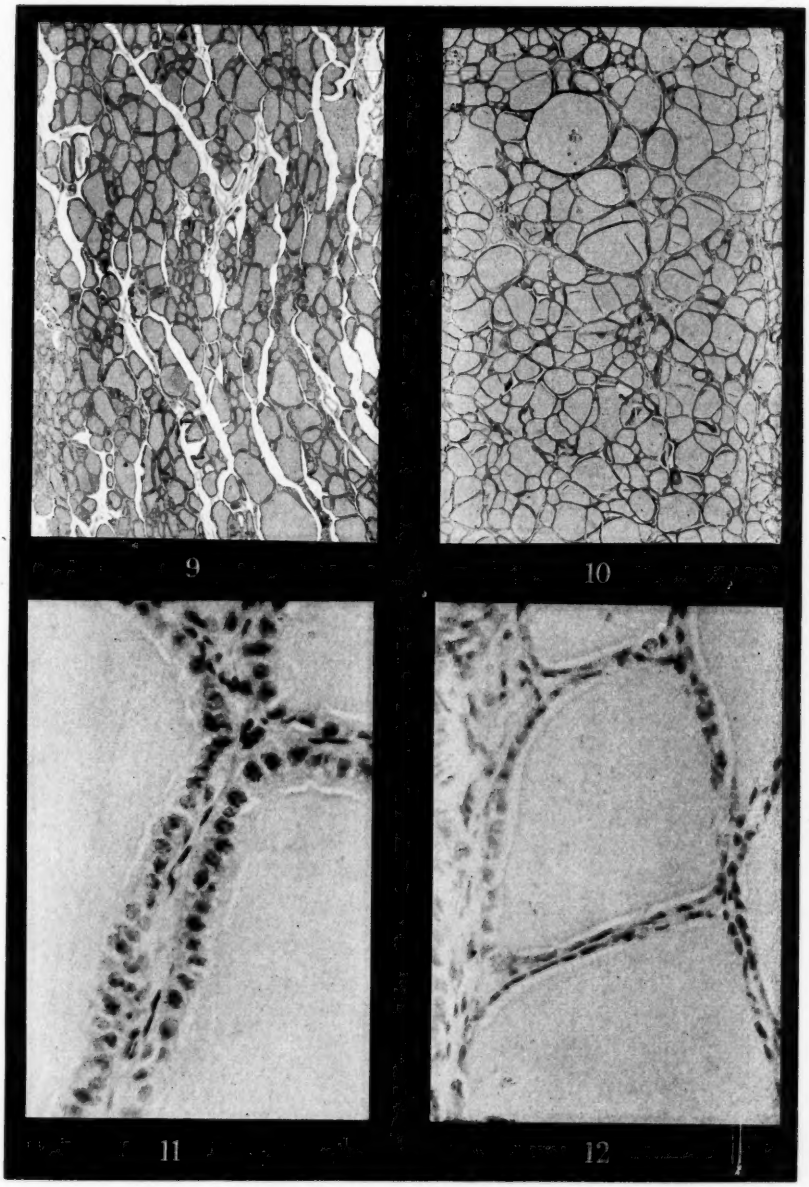

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Autopsy findings.

General. An interesting and unexpected finding when the treated animals were examined at abattoirs was first made by a butcher engaged in sawing the carcasses down the midline ; he volunteered the information that the resistance of the vertebrae to his circular saw was consistent with an age of 7–8 years (in contrast to the actual age of 2½ years, which he disputed). This estimate was confirmed independently when, during routine examination in another department of the abattoirs, the graders marked the cattle as " old cows." (See, however, under *Histological Examinations*). On the other hand, when the controls were butchered at the same abattoirs one week later, their age was not questioned and they were graded as " prime heifer beef." All animals were passed for human consumption.

Histological examinations :

1. Liver, spleen, kidney, adrenal gland, uterus, and ovary did not show any noteworthy differences between treated and control animals.

2. Mammary glands : Lactation in those animals which had calved at full term had been terminated at least 5 months before autopsy and, of course, had never really commenced in the 7 animals which had aborted. Therefore, advanced involution of the lobule-alveolar system had occurred, particularly among the controls, in which the sections revealed mostly a poorly developed duct system embedded in much adipose tissue and containing in some cases (C11, C12, C16) a certain amount of desquamated cells and secretion. On the other hand, in the treated animals, especially No. T22, T25, T26 and T31, there was much less adipose tissue and an advanced glandular development, revealed as an extensive proliferation of ducts, but without true alveolar development (Fig. 5, 6, 7, 8). Many ducts contained desquamated cells, fat globules and secretion.

3. Thyroid glands : Treated animals showed the expected change from normal cuboidal to low flattened epithelium acini (Fig. 9, 10, 11, 12). The acinar contents of the treated animals stained less deeply than in the controls, while the mean acinar cell height was significantly depressed, at the 1 per cent level, as the result of the treatment (Table III).

4. Pituitary glands from the three treated and from normal animals did not show any consistent differences.

5. Bone : The specimens available, which were limited to the osseo-chondral region of the spinous process of the 1st thoracic vertebrae, did not yield evidence to explain very satisfactorily the observed increased resistance of the vertebral columns to sawing. In some specimens there was a greater development of nutritive canals in the cartilage, but in the bone itself there was no obvious thickening of the trabeculae (Fig. 13, 14, 15, 16). It can only be assumed that the seat of the increased hardness must have been in the bodies of the vertebrae rather than in the spinous processes.

Thyroid iodine assays.—The values obtained from the assays for total iodine and for " thyroxine iodine " showed such wide individual fluctuations that the difference between the means for control and treated animals, for either characteristic, was not significant. (*Total iodine* : Control group, 2614μg./g. ± 282 ; treated groups, 2498 ± 230. *"Thyroxine iodine"* : Control group, 793 μg./g. ± 90 ; treated groups, 655 ± 73.) However, when the " thyroxine iodine " values were expressed as " percentage total iodine," the result of the comparison indi-

cated a depression, significant at the 1 per cent level, of this ratio in the thyroids of those animals which had received thyroxine treatment (Table III).

TABLE III.—*Acinar Cell Heights and "Thyroxine Iodine" (as per cent total iodine) of Thyroid Glands from Control and Thyroxine-treated Cows.*

Cow group.	Treatment.	Number of animals.	Acinar cell height. (μ.) Mean.	Acinar cell height. (μ.) S.E.	Thyroxine I (per cent total I). Mean.	Thyroxine I (per cent total I). S.E.
I	Controls	6	6·6	·25	30·4	1·2
II	Thyroxine injected	6	3·1	·25	25·9	1·2
III	,, ,, (large animals)	3	4·1	·35	25·9	1·7
II + III	Thyroxine injected (all animals)	9	3·5	·20	25·9	1·0

Comparison of Groups.

	Diff. +	S.E.	Diff. +	S.E.
I − (II + III)	3·1	·32	4·5	·16
Significance	P < ·01		P < ·01	

TABLE IV.—*Thyrotrophic Hormone Assays of Anterior Pituitary Powder (A.P.P.) from Control and Thyroxine-treated Cows, with Comparisons Using Standardized Thyrotrophic Hormone (T.H.) (34 G.P. units/mg.). Test Animal: 2-day-old White Leghorn Cockerel Injected Intraperitoneally Daily for 4 Days.* Statistical Treatment by Analysis of Variance Technique.* $\nu = 65$.

Chick group.	Treatment.	Number of test animals.	Daily dose of A.P.P. (mg.)	Daily dose of T.H. (units).	Thyroid acinar cell heights (μ). Mean.	Thyroid acinar cell heights (μ). S.E.
A	Controls (1·0 ml. saline).	8	—	—	2·26	·11
B	A.P.P. (treated)	10	2·0	—	2·34	·10
C	A.P.P. (treated) + T.H.	10	2·0	0·25	3·85	·10
D	A.P.P. (control)	11	2·0	—	4·76	·09
E	A.P.P. (control) + T.H.	12	1·0	0·25	4·89	·09
F	T.H.	12	—	0·25	3·91	·09
G	Controls (1·0 ml. saline)*	5	—	—	3·42	·14
H	A.P.P. (treated)*	5	2·0	—	3·12	·14

Comparison of groups.

Groups.	B–A. +	D–A. +	H–G. −	C–A. +	E–A. +	F–A. +	D–E. −	C–F. −	E–F. +
Diff.	·08	2·50	·30	1·59	2·63	1·65	·13	·06	·98
S.E.	·15	·15	·20	·15	·14	·14	·13	·13	·13
Significance	n	**	n	**	**	**	n	n	**

* Note in Groups G and H injections were continued for a further 6 days, i.e. 10 injections.
n = non-significant.
** = significant at 1 per cent level.

Chick assays of A.P.P. for thyrotrophic activity.—A.P.P. from treated cow T31 was tested against material similarly prepared from normal non-experimental cows and standardized hormone (T.H.) in a preliminary experiment involving 45 chicks in 7 groups. This established 2 mg. A.P.P. and 0·25 G.P. units of the standardized preparation as suitable daily dosages. Since, in the main experiment, pooled A.P.P. from the control group and from the matched treated group was used, it is of interest to note that in the preliminary experiment the mean acinar cell height of the chick group receiving A.P.P. from the single treated animal did not differ significantly from that of the control chick group, although the mean acinar cell height of the group receiving A.P.P. from normal cows was significantly elevated. Moreover, no significant difference was observed between the mean acinar cell height of chicks receiving A.P.P. (treated animal) supplemented by 0·25 units T.H., and that of those receiving the same dose of T.H. alone. Thus, under the conditions of the preliminary experiment, we could not demonstrate the presence of either thyrotrophic hormone or thyroid-inhibitory factor in A.P.P. from the thyroxine-treated animal.

The findings were similar when A.P.P. from each of the two groups of experimental cows was assayed with larger chick groups. The results of the assays and of the statistical treatment are set out in Table IV, from which the following conclusions may be drawn :

1. The result of thyroxine treatment was to depress the thyrotrophic potency of cow A.P.P., so that at a dosage level of 2 mg. for 4 or 10 days the presence of the hormone could not be detected.

2. At the same dosage level, A.P.P. from thyroxine-treated cows was not able to depress the effect on the chick's thyroid of 0·25 G.P. units of thyrotrophic hormone per day concurrently administered.

3. On the other hand, an equivalent amount of A.P.P. from control cows did contain a significant quantity of thyrotrophic hormone, the probable content (comparison of Groups D and E) being of the order of 0·25 G.P. units per mg., i.e. 250,000 units per kg. (Junkmann, 1936).

It is of interest to note that iodine could not be detected in 10 mg. samples of A.P.P. from thyroxine-treated animals. Thus the failure to establish thyrotrophic activity in this material could not be correlated with a depressing effect on the chick's thyroid of iodine-containing contaminants.

DISCUSSION.

The results of the investigation have demonstrated that the prolonged administration of thyroxine to cows has a marked effect on the functions of the thyroid and pituitary glands of this animal. The reduction in the thyroid acinar cell height and in the proportion of " thyroxine " to total iodine within the gland may be related to a direct effect of the injected thyroxine or, more probably, to an indirect effect mediated by the pituitary. By chick assay we were unable to demonstrate the presence of thyrotrophic hormone (or of a thyroid-inhibitory factor) in the pituitary glands of our thyroxine-treated animals ; the validity of these findings is enhanced by the fact that in two of the three relevant chick group comparisons the mean acinar cell heights of the control chick groups were neither at the minimal value (that of Group A, Table IV), nor on the plateau of a dose-response curve for thyrotrophic effect (Table IV : Group H v. Group G ; Group C v. Group F). The probable zero level of thyrotrophic hormone in the

A.P.P. of our treated animals is consistent with an inhibition of production of the hormone or with its more rapid secretion into the blood stream. The generally accepted view (Salter, 1940) is that thyroid hormone circulating in excess has a depressing effect on the production of the pituitary hormone. This regulatory mechanism is, moreover, in line with the observation of Blaxter (1946) that cessation of the treatment of lactating cows with iodinated casein led, after a few days, to a sudden and severe drop in milk production. With both the pituitary and thyroid glands at low functional levels, it might be expected that a hypometabolic state would intervene between the cessation of treatment and the return to a normal pituitary-thyroid relationship.

The absence of a thyroid-inhibitory factor in the pituitary glands of the thyroxine-treated animals is not in accord with the observation of Reforzo-Membrives (1943). This author used relatively larger thyroxine-equivalent dosages for his rats, but the material, thyroid powder, was given by mouth and was possibly incompletely absorbed (Krayer, 1928). The total quantity of thyroxine received by our animals during the 70 days of the experiment works out at 11·5 mg. mean daily intake, slightly in excess of that found by Schultze and Turner (1945) as necessary to maintain the milk supply in lactating cows " chemically thyroidectomized " by treatment with thiourea. As discussed below, the effect of our daily dose has been reflected to a marked extent in the average clinical picture of the treated animals, and it might, therefore, be assumed that, if the production of a thyroid-inhibitory factor by the pituitary gland is of physiological significance, evidence for its existence should have been provided from our results. Our negative finding is in accord with those of Adams and Jensen (1944) and Purves and Griesbach (1946). The former authors actually injected their mice with a larger total dose of thyroxine (considered for equivalent body weights) than that administered orally as thyroid powder to rats by Reforzo-Membrives during a similar period.

The amount of thyroxine administered was sufficient to produce marked effects upon weight gains, cardiac frequency, respiratory frequency and excitability. In the extensive experiment of Blaxter (1946), where lactating cows on a definite ration were used, a mean weight loss of 18 lb. was found. That our treated animals gained weight, although only about 30 per cent of that gained by the controls, was doubtless due to the provision of high class nutriment *ad libitum* and to the absence of lactation. It is, therefore, not surprising that there was no very marked change in the concentration of serum protein (as judged by specific gravity) or of antibody. The depressing effect of thyroxine upon weight gains has its parallel in a significant effect in preventing the rise of the S.G. of the blood (probably referable to an increase in red cell count) which occurred in the control animals during the period of the experiment ; the effect of thyroxine upon red cell count may thus be in part nutritional in origin, i.e. related to the inability of the animal to consume or to assimilate sufficient extra nutriment to offset the increased metabolism. It is, however, of interest that Wilson (1944) found that no significant change occurred in the red cell count of adult rats given standard diet *ad libitum*, and injected intramuscularly with thyroxine at the rate of 1 mg. every 2–3 days for 11–30 days. The basal respiratory and cardiac frequencies of adult cattle, i.e. " the number per minute in a mature animal in a state of rest and free from mental excitement " (Dukes, 1937) is given variously in the literature from as low as 8 to as high as 30 for respirations and from 40

to 80 for heart beats. Our findings were a maximum mean respiratory frequency of 57 for controls and 77 for treated, and a maximum mean cardiac frequency of 76 for controls and 97 for treated animals. The general high level of both values, even in the control animals in this experiment, is probably related to the fact that the animals were not housed in stalls, but were brought in from the field for clinical examinations; they are more comparable with, though even higher than, the values recorded by Blaxter (1946) for the mean cardiac frequency in mature Shorthorn lactating cows in commercial dairies, no doubt under somewhat similar conditions, namely, 71·3 for controls and 82·8 for thyroprotein-treated.

Few, if any, records appear to exist on the influence of thyroxine upon the non-lactating involuted mammary gland in the non-pregnant cow. Indeed, according to Turner (1939), no systematic study even of involution of the udder in dairy cattle which might have formed a basis for assessing our findings has yet been made. Petersen (1944), reflecting general opinion, considers that the influence of thyroxine upon lactation is undoubtedly due to its effects upon metabolism; but this refers to the lactating animal. Our observations indicate a marked effect upon udder development, revealed macroscopically as enlargement of the udder, turgidity of the teats and increased secretion and microscopically as extensive proliferation. Although some of the animals had been infected with virulent *Br. abortus*, as a result of which some of the microscopic lesions described by Ridala (1936) were present in the udders (e.g. leucocytic infiltration), it is not considered that the duct proliferation had any relationship to this. The only relevant observations of which we are aware are those of Gardner (1942) who found that consumption of desiccated thyroid by intact male mice (but not by castrated males) 40–60 days old for 17–49 days was followed by marked growth of the mammary glands due to proliferation of ducts. We have subsequently had access to a relevant paper by Dyrendahl (1946). This author administered 10 g. iodocasein per day to a normal heifer 14 months old for 10 months, and noted a gradual increase in udder volume after 1½ months' treatment until a stage of development was reached comparable to that usually seen in a first-calf animal. In 270 days a milk yield of 800 l. was obtained. The illustration accompanying the paper shows a moderate udder development, with well-developed turgid teats, such as we found in our older thyroxine-treated animals.

The observed effects upon the hardness of the vertebral column were not expected, and, therefore, it was not possible to examine this aspect very thoroughly. It is hoped to do so in the future.

SUMMARY.

1. Nine Shorthorn cows in two groups were each injected with a total of 805 mg. thyroxine during 10 weeks, while six animals of the same age (about 2½ years) served as controls. Clinical and post-mortem findings were recorded.

2. Thyroid function was depressed, as shown by a significant lowering of the acinar cell height and of the ratio of thyroxine to total iodine. Significant amounts of thyrotrophic hormone were shown by chick-thyroid assay to be present in the anterior pituitaries of the control animals, but it could not be detected in the glands of those which had been treated. A thyroid-inhibitory factor could not be demonstrated in the anterior pituitaries of the animals receiving thyroxine.

3. As the result of the treatment there was a depression of weight gain and

of the specific gravity of the blood, while the respiratory and cardiac frequencies were elevated.

4. The adrenal, ovarial and pituitary glands and the livers, spleens, kidneys and uteri of the thyroxine-treated animals were of normal histological appearance. However, the spinous process of the 1st thoracic vertebrae showed some change, while the mammary glands were characterized by an advanced glandular development with reduction in the amount of adipose tissue.

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RIFT VALLEY FEVER. ISOLATION OF THE VIRUS FROM WILD MOSQUITOES.*

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IN their original article announcing the discovery of Rift Valley fever virus, Daubney and Hudson (1931) expressed the opinion that the virus is transmitted by mosquitoes, and brought forth a considerable amount of circumstantial evidence to support this view. Later they (Daubney and Hudson, 1933) explored this possibility further, and found that the virus could be recovered from mosquitoes several days after an infective feeding by inocualtion of a suspension of the insects into susceptible animals. However, up to this time, the virus has not been isolated from naturally infected mosquitoes, and there has been no report of the successful transmission of the disease by the bite of naturally or experimentally infected mosquitoes.

In 1944 we were engaged in co-ordinated field and laboratory investigations on the epidemiology of yellow fever, one aspect of which was an attempt to identify the vector of that disease in primeval forest uninhabited by humans. Large numbers of mosquitoes were captured (Haddow, Gillett and Highton, 1947), and suspensions of them were inoculated into mice and monkeys (Smithburn and Haddow, 1946). Over 60 species of the insects were included in the captures (Haddow, Gillett and Highton, 1947), and some grouping of species was resorted to for the preparation and inoculation of the suspensions. During the course of this study Rift Valley fever virus was isolated 6 times from mosquitoes included in these forest catches. The present communication is an account of these isolations of the virus. A report of the successful experimental transmission of the disease by one of the groups of mosquitoes from which the virus was isolated is given in a separate paper (Smithburn and Haddow, in press).

MATERIALS AND METHODS.

The methods employed in the mosquito catches (Haddow, Gillett and Highton, 1947 ; Haddow, 1945) and in the preparation of mosquitoes for inoculation of animals (Smithburn and Haddow, 1946) have been described in detail previously and need be repeated here only briefly.

Mosquito catches.

The insects were captured at ground level in forest by a squad of about 20 experienced African youths who moved singly through the forest, each provided with a supply of glass tubes, and collected the mosquitoes in the tubes as they alighted to feed. The tubes were then lightly plugged with cotton and were transported to the field laboratory, where the mosquitoes were

sorted and identified. They were then released into separate Barraud cages by groups or species, and fed on bananas and water until such time as the whole catch was sent to Entebbe. For this journey the Barraud cages were placed in ventilated wooden cases and were covered above with layers of wet cotton.

Catches were made daily on five consecutive days (Tuesday to Saturday). The mosquitoes were sent to Entebbe on Sunday and inoculations were made on Monday. All mosquitoes with which we are here concerned were caught in uninhabited forest at Mongiro, Bwamba County, Toro District, in western Uganda. The 275-mile journey to the laboratory at Entebbe did not give rise to excessive mortality in the mosquitoes.

Inoculations.

On arrival at the laboratory at Entebbe the mosquitoes were inspected and the number of dead in each species or group was recorded. In most instances these were used along with the living, but in a few instances dead insects were discarded. The living insects were killed with chloroform and were then triturated in a sterile porcelain mortar. When the number of insects was very large, sterile powdered pyrex glass was employed to facilitate the grinding. After thorough grinding, sufficient 10 per cent normal serum in physiological saline solution (hereafter referred to as diluent) was added to make a good suspension. This was spun in the angle centrifuge for 20 minutes to one hour at about 3000 r.p.m., and the supernate was removed. A small portion of this supernate was passed through a Seitz E.K. pad, and the filtrate was used for intracerebral inoculation of a group of 6 mice. Another group of mice was inoculated with unfiltered supernate, and the remainder of both the unfiltered and Seitz-filtered portions were inoculated subcutaneously into a normal rhesus monkey.

Observations of animals.

Inoculated monkeys were observed at frequent intervals, and their temperatures were taken twice daily. Whenever a monkey had a temperature of 104° F. or higher, or when there was other reason for doing so, the animal was bled and its serum was subinoculated intracerebrally into mice. Portions of pre-inoculation serum were compared with post-inoculation sera in tests for immunity whenever virus was isolated from mosquitoes with which the monkey concerned had been inoculated.

Mice were examined daily in the forenoon, and brain passages were made from any which appeared ill. Intracerebral passage of brain is neither the optimal route nor tissue for Rift Valley fever virus, but as we were engaged in yellow fever investigations the identity of the virus with which we were dealing was not immediately discovered. Tissues of some sick and dying mice were saved for histological studies, and it was by this means that the identity of the virus was first discovered.

Isolations of the Virus.

The catch which yielded the first strains of Rift Valley fever virus was made from the 18th to the 22nd of April, 1944, and included 4310 mosquitoes, of which 3860 were sent to Entebbe. The catch included large numbers of *Aëdes*

(*Aëdimorphus*) *tarsalis* Newst. group, and *A.* (*Banksinella*) *circumluteolus* Theo., which were kept as separate species. All other mosquitoes were grouped generically and so used for inoculation. Separate groups of mice were inoculated with suspensions of *Anopheles*, *Culex*, *Taeniorhynchus*, *Eretmapodites*, *Aëdes*, *A. tarsalis* and *A. circumluteolus*. Separate normal rhesus monkeys were inoculated with all these except *Culex*. Yellow fever virus was isolated from the *Aëdes* mosquitoes (Smithburn and Haddow, 1946) and Rift Valley fever virus from the *A. tarsalis* and the *Eretmapodites*, as will be seen from the following accounts.

Strain 1.—The 2307 mosquitoes of the *A. tarsalis* group (lot 26) were used to prepare a suspension with which 6 mice and one rhesus monkey were inoculated. The mice received Seitz-filtered suspension intracerebrally, and rhesus 395 received subcutaneously 10·3 ml. of the unfiltered suspension, the total volume of which was originally 17 ml.

Two of the inoculated mice sickened, and brain passages were made from them without positive result. The other 4 mice remained well.

The rhesus, No. 395, had fever 4 days after inoculation; it was bled in the morning and afternoon, and mice were inoculated intracerebrally with its serum on each occasion. Rhesus 509 was also inoculated subcutaneously with both specimens of serum. Both groups of subinoculated mice were well 24 hours after inoculation, but 10 of the 12 mice were dead by the morning of the second day. Subinoculations of brain were made from the 2 sick mice remaining, and two lines of virus, each readily transmissible in series, were established in mice. These were carried through 24 passage generations.

Rhesus 395 had no further fever or other signs of illness, but was subsequently found to have developed specific antibody against the virus isolated. Rhesus 509 had neither febrile nor other objective reaction to the inoculation of virus-containing serum from rhesus 395, but likewise developed antibody against the virus (Table I).

It is noteworthy that this virus strain failed to become established directly in mice following intracerebral inoculation with Seitz-filtered mosquito suspension, but did so readily after a single passage through a rhesus monkey.

Strain 2.—441 *Eretmapodites* spp. mosquitoes (lot 28) taken in the same catch as the *A. tarsalis* (lot 26) were used to prepare a suspension from the Seitz filtrate of which a group of mice was inoculated intracerebrally. Rhesus 398 received a subcutaneous inoculation of 4 ml. of unfiltered supernate representing 2/3 of the original suspension.

All the mice remained well during three days, but on the fourth day 3 were dead and 2 sick. Brain passages were made from both the latter. The second passage mice remained objectively well for two days, but were all dead on the morning of the third day. A third passage from a dead mouse was successful and gave continuity to a line of virus transmissible in series, with the survival time thereafter averaging less than 72 hours from the time of inoculation.

Rhesus 398 had no objective reaction to inoculation of the mosquito suspension. However, on the morning when the mice receiving the same inoculation first showed evidence of infection, Rhesus 398 was bled and its serum was subinoculated into mice. Two days later 2 of the mice were dead and another, which was sick, was sacrificed for subinoculation. The remaining 3 mice were dead on the third day. Subinoculation from the sick mouse gave rise to a line

of virus which was regularly transmissible in series, and which proved to be identical with the virus isolated directly in mice.

Rhesus 398 was subsequently found to have developed neutralizing antibody against this virus (Table I) following the inoculation of the *Eretmapodites* mosquitoes.

The 2 lines of this strain were carried through 19 and 17 passage generations respectively. Two lots of the virus were successfully preserved by drying while frozen ; further batches were preserved in glycerine, likewise with success.

Strain 3.—Strains 3 and 4 were isolated from mosquitoes caught between May 16 and May 20 in the same forest area as the mosquitoes from which strains 1 and 2 were isolated. No catches were made in the interval between April 22 and May 15, so that the catch which started on May 16 was the next in succession.

A suspension was prepared from 60 *Aëdes* (*Stegomyia*) *de-boeri* ssp. *de-meilloni* Edw. (lot 33) in 4·0 ml. of diluent, and intracerebral inoculations of filtered and unfiltered portions were made into adult mice, and of the Seitz filtrate into 4-day-old mice. Rhesus 396 received the remaining 2·4 ml. of unfiltered supernate subcutaneously.

Three days after these inoculations all the mice receiving unfiltered suspension were dead, though all had appeared well on the second day. Passages were made from brains of 2 dead mice, with the result that 2 lines of virus were established and carried in series through 10 generations. As with the previous strains, the survival time in mice receiving 10 per cent brain suspension intracerebrally (unfiltered or filtered) averaged less than 72 hours.

The adult mice receiving Seitz-filtered mosquito suspension remained well ; a " blind " passage made from one of them on the third day was unsuccessful. Of the infant mice receiving the Seitz filtrate intracerebrally 1 died. Two others sickened and were sacrificed for passage, but virus was not isolated from them. This failure of mice receiving the Seitz filtrate of the mosquito suspension to become sick may have been due to a low content of virus in the mosquitoes, with resultant failure of the virus to pass the filter.

Rhesus 396 had no clinical reaction as a result of the inoculation of mosquito suspension, but was subsequently found to have developed neutralizing antibody against the virus isolated in mice.

Strain 4.—942 *Eretmapodites* spp. (lot 52) of the catch of May 16 to 20 were used to prepare a suspension, filtered and unfiltered portions of which were inoculated intracerebrally into mice. No inoculation of this suspension was made into a monkey.

All the mice which received the unfiltered suspension of mosquitoes were dead 24 hours after inoculation. These deaths were believed to be due to bacterial contamination, and no passage was made.

Of the 6 mice which received the Seitz filtrate of this lot of *Eretmapodites*, 1 was dead on the first day, 3 on the third day and 1 on the sixth day. Passage was made on the third day with the brain of a mouse which appeared well. This was successful, and the virus was thereafter easily transmissible with either unfiltered brain suspension or Seitz filtrate. It was transmitted in series through 10 passages, the mean survival time from the second passage onward being less than three days.

Strain 5.—Strains 5 and 6 were isolated from mosquitoes caught May 23 to 27, 1944, in the same area as the mosquitoes which gave rise to strains 1 to 4.

This catch included 2326 *Aëdes tarsalis* group mosquitoes (lot 60). Unfiltered supernate of a suspension of these mosquitoes in 20 ml. of diluent was inoculated intracerebrally into a group of adult mice, and the Seitz filtrate of the same suspension was inoculated intracerebrally into a group of adult and a group of 3-day-old mice. The remainder of the suspension, 16 ml., was inoculated subcutaneously into Rhesus 535.

All mice receiving the unfiltered suspension were dead 24 hours after inoculation. The deaths were believed due to bacterial contamination, and no passage was made. The adult and infant mice which received the filtered suspension remained well.

Rhesus 535 had fever on the third and fourth days after inoculation with the unfiltered suspension of *Aëdes tarsalis* group, lot 60, and died on the fifth day. Death was caused, at least in part, by a huge abscess at the site of inoculation. Specimens of serum taken at the time the animal had fever on the third and fourth days were inoculated intracerebrally into groups of mice. A Seitz-filtered suspension of the liver of Rhesus 535 was inoculated intracerebrally into mice, and subcutaneously into Rhesus 542. Ten of the 12 mice inoculated with the serum of Rhesus 535 were dead within 48 hours of inoculation. Another mouse which was sick on the second day was sacrificed for passage. The twelfth mouse was dead at 96 hours. Passage from the sick mouse on the second day gave rise to a line of virus which was readily transmissible in series, the average survival time being three days or less in each of the 38 serial passages made.

Inoculation of mice with filtered and unfiltered suspension of liver taken from Rhesus 535 at autopsy also resulted in isolation of the virus from that tissue. This line of virus was not passed in series, but in pathological studies the lesions induced by it were found to be identical with those induced by the virus isolated from the serum of Rhesus 535.

Rhesus 542 had no clinical reaction as a result of inoculation with the filtered suspension of liver of Rhesus 535, but it developed neutralizing antibody in its serum, and thus confirmed the recovery of virus in mice from the serum and liver of Rhesus 535.

The failure of mice receiving the Seitz-filtered suspension of *Aëdes tarsalis* (lot 60) to sicken may again indicate a low content of virus in the mosquitoes. Had Rhesus 535 not had a large subcutaneous abscess, believed largely responsible for its fever, illness and death, this strain of virus might not have been isolated.

Strain 6.—The *Eretmapodites* spp. (lot 71) used for inoculation on May 29 included 347 mosquitoes. Unfiltered and filtered portions of the suspension of these mosquitoes were inoculated intracerebrally into mice, but no inoculation was made into a monkey. Adult mice which received the unfiltered suspension appeared well on the second day, but all were dead on the third day. Passages from the brains of two of the dead mice were successful, and the line of virus so established was carried through 44 passage generations.

The 6 adult mice which received Seitz-filtered mosquito suspension remained well. However, each of a group of three-day-old mice inoculated intracerebrally with the Seitz filtrate sickened and died, and passage from the brain of a sick mouse of this group was successful. This line was likewise transferred in series through 44 mouse generations; the mean survival time in groups receiving either of these lines of virus was never more than 2·1 days. Passage of Seitz-filtered brain from infected mice was repeatedly and invariably successful.

There were no survivors in any of the groups of mice receiving inoculations in either series.

The numbers of mosquitoes used for inoculation from the three productive catches and the animals into which they were injected are shown in Table III.

Identification of the Virus.

Histological studies.

Tissues of a sick mouse of the second passage group receiving Strain 1 virus intracerebrally were fixed in 10 per cent formol-saline, and stained sections were prepared. Despite the fact that the mice were inoculated intracerebrally, sections of the brain of this mouse showed no lesions attributable to a virus disease. The heart and lungs appeared normal, both in the gross and microscopically. The kidneys, spleen and liver exhibited moderate congestion. The liver was brownish-red in colour, normal in size and slightly less firm than normal liver. Microscopically the principal lesions involved the liver. These consisted of massive pan-lobular necrosis of hepatic cells, congestion and haemorrhage, infiltration of sinusoids with polymorphonuclears and mononuclears, " hyaline " acidophilic degeneration of portions of the cytoplasm of degenerating hepatic cells, and the appearance of acidophilic inclusion bodies in the nuclei of some degenerating parenchymal cells.

The examination of tissues of mice receiving other strains of the virns yielded similar results. Moreover, the liver of Rhesus 535 exhibited the same qualitative abnormalities, although the hepatic lesions in this animal were less intense than those in the mice examined.

The histological studies in the second passage mouse receiving Strain 1 virus gave the first information of the identity of the virus. The lesions observed very closely resembled those induced in liver tissue by Rift Valley fever virus as described by Daubney and Hudson (1931) and Findlay (1933). Immunological studies were undertaken to determine the correctness of this tentative identification.

Immunological studies.

Early in the study of these new strains of virus it was found that they are highly pathogenic for mice by intraperitoneal inoculation. It was then found that an effective mouse protection (virus-neutralization) test could be performed by the intraperitoneal injection of 0·06 ml. per mouse of a mixture of 0·5 ml. of the serum to be tested and 0·25 ml. of a 10 per cent or weaker dilution of brain or liver of an infected mouse. As the histological studies had indicated the probability that the virus was that of Rift Valley fever, a supply of specific bovine antiserum against this virus was procured.*. This serum protected mice completely against virus Strain 6 in a preliminary exploratory experiment. We also had in hand the convalescent serum of a laboratory technician who suffered an attack of Rift Valley fever in New York (Dr. S. F. Kitchen's (1934) Case No. 1). By this time, " convalescent " sera were available from 5 monkeys inoculated either with mosquito suspensions or with serum or liver suspension of monkeys which received mosquito suspensions from which strains of virus were

* From J. R. Hudson, of the Veterinary Research Laboratory at Kabete, Kenya, whose generosity in supplying this is gratefully acknowledged.

isolated. A protection test was set up to test these sera, together with suitable controls, against Strain 2 virus (from *Eretmapodites*). The results of this test are shown in Table I.

TABLE I.—*Summary of Results of an Intraperitoneal Protection Test Showing that Virus Strain 2 is Rift Valley Fever Virus and that Convalescent Sera of Inoculated Monkeys Neutralize the Virus.*

Serum.	Virus dilution, log.	Fate of mice. Died.	Fate of mice. Lived.	Titre of virus.	L.D.$_{50}$ of virus.
Pooled preinoculation sera of Rhesus 396 and 398	3	6	0	6,600,000	6600
	4	6	0		660
	5	4	2		66
	6	5	1		6·6
	7	4	2		0·6
	8	0	6		..
Rhesus 398, preinoculation	2	6	0		66,000
,, ,, convalescent	2	0	6	<100	,,
Rhesus 265, immune yellow fever and Semliki Forest virus*	2	6	0	..	,,
Rhesus 509, convalescent	2	0	6	<100	,,
Normal Ox 1	2	6	0	..	,,
,, ,, 2	2	6	0	..	,,
Rift Valley fever immune ox	2	0	6	<100	,,
Non-immune human	2	6	0	..	,,
Human immune Rift Valley fever	2	0	6	<100	,,

* Rhesus 265 was immune to both yellow fever and Semliki Forest (Smithburn and Haddow, 1944) viruses as a result of experimental procedures.

This experiment showed not only that convalescent sera of rhesus monkeys 398 and 509 contained specific neutralizing antibody, but, more important, that Rift Valley fever immune ox serum and the serum of a human convalescent from the disease gave complete neutralization of no less than 66,000 L.D.$_{50}$ of virus. The titre of virus was determined by the method of Reed and Muench (1938), and the end-point dilution is regarded as containing one L.D.$_{50}$. The identity of Strain 2 was thus proved. An experiment was then undertaken to determine whether the 6 strains are immunologically identical. Pre-inoculation and convalescent sera of monkeys receiving suspensions of mosquitoes from which Strains 1 and 2 were isolated or subinoculation of serum from a monkey infected with strain 1 were tested against 1 per cent suspensions of each of the 6 strains of virus. The results of the test are summarized in Table II.

In the foregoing cross-neutralization test no titrations of virus were done. However, in other experiments being done at this time the titres were consistently over 1 in 100,000, so it is reasonable to suppose that the test dose of each virus in this experiment was at least 1000 M.L.D. The results show that the pre-inoculation sera contained no protective antibody against any of the strains, that convalescent sera which contained neutralizing antibody against the homologous strains (1 and 2) neutralized the other 4 strains as well, and therefore that the 6 strains are identical.

TABLE II.—*Intraperitoneal Cross-protection Tests with the 6 Strains of Virus, each Tested against Preinoculation and Convalescent Sera of Monkeys Inoculated with Strains 1 and 2.*

Serum of Rhesus.			Tested against 1 per cent virus, strain.	Fate of mice.	
Number.	Time taken.	Inoculated with Strain.		Died.	Lived.
395	Pre-inoculation	1	1	6	0
398	,,	2		6	0
509	,,	1		6	0
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6
395	Pre-inoculation	1	2	6	0
398	,,	2		4	2
509	,,	1		5	1
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6
395	Pre-inoculation	1	3	6	0
398	,,	2		6	0
509	,,	1		6	0
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6
395	Pre-inoculation	1	4	6	0
398	,,	2		6	0
509	,,	1		6	0
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6
395	Pre-inoculation	1	5	6	0
398	,,	2		6	0
509	,,	1		6	0
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6
395	Pre-inoculation	1	6	6	0
398	,,	2		6	0
509	,,	1		6	0
395	Convalescent	1		0	6
398	,,	2		0	6
509	,,	1		0	6

Other studies carried out at this time, but which need not be described in detail, showed that the virus is not neutralized by immune sera against the Semliki Forest (Smithburn and Haddow, 1944), Bunyamwera (Smithburn, Haddow and Mahaffy, 1946), yellow fever or eastern or western equine encephalomyelitis viruses.

Epidemiological Investigations.

This being the first occasion on which Rift Valley fever virus was isolated from naturally infected mosquitoes, it was deemed advisable to attempt to discover—

1. Whether other mosquitoes were involved in the cycle of infection.
2. Whether humans became infected.
3. Whether wild animals were attacked by the virus.

Investigations of these points were undertaken. The results, although largely negative, nevertheless shed some light on this outbreak of Rift Valley fever.

The role of other mosquitoes.

Mosquito catches were in progress at Mongiro, Bwamba, from January to July 8, 1944. Series of catches were made on 5 consecutive days in January, February and March. Two 5-day catches were made from April 4 to 8 and 18 to 22. From May 16 until May 27 catches were made on 5 days of each week. Ten 24-hour catches were made between May 29 and July 8. No virus was isolated from the 12,557 mosquitoes caught prior to April 18, nor from the 3042 taken and used for inoculation after May 27. Each of the three catches made between April 18 and May 27 yielded strains of Rift Valley fever virus, so it can be stated with certainty that Mongiro was a focus of virus activity during that period.

Many species and genera were included in the 18,884 mosquitoes of the productive catches, and during the period of virus activity suspensions of 50 lots were inoculated into 26 different non-immune rhesus monkeys and into mice, or, in the case of 9 lots, into mice only. The numbers of each genus or species of mosquitoes from each of the productive catches, suspensions of which were inoculated into experimental animals, are shown in Table III, together with the animals into which they were inoculated.

Three of the rhesus monkeys used in these experiments died, one (No. 421) of yellow fever, one (No. 535) with sepsis and Rift Valley fever, and one (No. 525) from haemopericardium following cardiac puncture. Monkeys 395, 396, 398 and 509 became immune as a result of inoculations made in conjunction with the aforementioned isolations of virus. Sera of the other 19 monkeys inoculated with suspensions of mosquitoes during the period of virus activity at Mongiro were all tested for antibody against Rift Valley fever, the samples of blood being taken at least 35 days after the May 29 inoculations. None of the 19 monkeys had protective antibody in its serum. Further, although each generic or species lot was inoculated into mice, Rift Valley fever virus was not isolated from any of the lots other than those mentioned. It may, therefore, be concluded that no species included in the catches contained virus in demonstrable quantities other than those from which virus was isolated.

Search for immunity in humans.

Twenty-five African youths who had participated in the field work as mosquito catchers at Mongiro were bled and their sera were tested against virus strain 2. None of their sera contained neutralizing antibody.

As previously stated, Mongiro, the locality in which the virus-containing mosquitoes were captured, is an uninhabited area on the northern boundary of

TABLE III.—*Mosquitoes of the Three Productive Catches, showing Groupings used for Inoculations and Animals into which they were Injected.*

Species or generic grouping.	Catch April 18–22; inoculations April 24.		Catch May 16–20; inoculations May 22.		Catch May 23–27; inoculations May 29.	
	Number of mosquitoes.	Rhesus Number.	Number of Mosquitoes.	Rhesus Number.	Number of Mosquitoes.	Rhesus Number.
Anopheles spp.	567	441	1802	Mice only	267	Mice only
Culex spp.	209	Mice only	423	,, ,,	186	,, ,,
Eretmapodites spp.	441*	398†	942*	,, ,,	347*	,, ,,
Taeniorhynchus spp.	20	396	145	,, ,,	42	,, ,,
Aëdes (*Finlaya*) *ingrami* Edw.	..	..	24	440	14	541
A. (*Stegomyia*) *aegypti* L.	..	..	6	440	..	..
A. (*S.*) *apicoargenteus* Theo.	3	421	93	395	22	533
A. (*S.*) *fraseri* Edw.	..	..	4	440	..	..
A. (*S.*) *de-boeri* ssp. *de-meilloni* Edw.	12	421	60*	396†	33	531
A. (*S.*) *africanus* Theo.	3	421‡	133	398	19	532
A. (*S.*) *luteocephalus* Newst.	..	..	2	440	..	..
A. (*Aëdimorphus*) *capensis* Edw.	..	..	..	..	3	541
A. (*A.*) *haworthi* Edw.	1	421	16	441	9	536
A. (*A.*) *argenteopunctatus* Theo.	4	421	1	509§	7	530
A. (*A.*) *mutilus* Edw.	1	421	13	519	..	..
A. (*A.*) *domesticus* Theo.	..	..	5	440	5	541
A. (*A.*) *tarsalis* Newst. group	2307*	395†	6647	520	2326*	535‖
A. (*A.*) *abnormalis* Theo. group	9	421	..	..	..	..
A. (*A.*) *lamborni* Edw.	3	421	5	522	2	538
A. (*A.*) *cumminsi* Theo.	13	421	26	524	16	537
A. (*A.*) *natronius* Edw.	11	421	228	525	58	539
A. (*Banksinella*) *circumluteolus* Theo.	236	440	408	440	608	541
A. (*B.*) *taeniarostris* Theo.	9	421	14	527	4	540
A. (*B.*) *palpalis* Newst.	11	421	28	526	15	534
A. (*Dunnius*) sp.n.	..	..	11	440	5	541

* Rift Valley fever virus isolated from these lots.
† Monkey became immune to Rift Valley fever as result of inoculation.
‡ Yellow fever virus isolated in Rhesus 421, and this mosquito is the most probable vector.
§ Immune to Rift Valley fever at time of this injection as result of previous inoculation with virus-containing serum of rhesus 395.
‖ Animal died. Subinoculation to Rhesus 542 resulted in the latter becoming immune to Rift Valley fever.

the Semliki Forest. Adjoining the forest on the north are the Semliki Plains, which are populated. A serum-collecting programme was carried out in the plains area in August, 1944, for the purpose of a yellow fever post-vaccination immunity survey. The residues of these sera being available, 20 from adults and 20 from children under 15 years of age were tested against virus strain 2. Three of these, all from adults, gave inconclusive results, and could not be retested owing to insufficiency of serum. The other 37 contained no protective antibody.

Eighty-nine sera from as many humans residing in other localities in Bwamba, south and east of the forest, were also tested. Forty-four of these were from adults and 45 from children under 15. Two adults residing in different localities, the nearest 12 miles from Mongiro, had protective antibody in their sera, while all the others were non-immune. For comparison with these, sera of 40 adults residing at Old Entebbe were also tested, with the result that the serum of 1 was fully protective, while the others contained no antibody against Rift Valley fever virus.

From these results it may be concluded that humans were not actively, if at all, involved in the outbreak of the disease at Mongiro in 1944. This is not surprising, as the only persons much exposed to infection there were those engaged in the mosquito catches. The absence of immunity in the latter may indicate that the insect vector operative in this outbreak does not feed actively on humans when its natural hosts are present, as must have been the case at Mongiro.

Tests for immunity in wild animals.

Sera of 72 wild monkeys belonging to 9 species taken in Bwamba, 30 before, 1 during and 41 after the period of activity of the virus at Mongiro, were tested for capacity to neutralize the new strains of virus. None of these contained neutralizing antibody, although 15 of the monkeys came from the Mongiro area and 10 of these were taken during or after the virus isolations. It therefore seems likely that monkeys were little, if at all, involved in this or any recent cycles of infection with Rift Valley fever virus in the Bwamba lowlands.

Two non-immune rhesus monkeys were stationed at Mongiro as yellow fever sentinels during April and May, 1944. Neither acquired immunity to Rift Valley fever virus during this period.

Sera from a red forest buffalo (*Syncerus nanus* (Boddaert)) and a waterbuck (*Kobus defassa* ? ssp.) shot in 1946 near Mongiro were tested for immunity to Rift Valley fever. Both animals were adults and quite old enough to have been alive at the time of the virus activity in 1944, yet both sera were non-protective.

It is perhaps worthy of note that a dead buffalo was encountered in the middle of the mosquito-catching area during the first of the three catches from which virus was isolated. A sick buffalo calf was also seen daily on the road nearby for a period of about ten days, after which it disappeared. It is possible that these animals were infected with Rift Valley fever, but by the time the virus was isolated it was impossible to obtain any information on the point.

Entomological Note on Mosquitoes from which Virus was Isolated.

Rift Valley fever virus was isolated from a lot of 2307 *Aëdes* (*Aëdimorphus*) *tarsalis* Newst. group, and subsequently from another lot of 2326 of the same group. At Mongiro this group consists of roughly equal numbers of *A.* (*A.*)

tarsalis Newst. and *A.* (*A.*) *albocephalus* Theo., the females of which cannot be distinguished reliably in life. There is therefore no possibility of telling from which of these species the virus was isolated, or whether both may have been involved.

Virus was also isolated from a lot of 60 *Stegomyia*, belonging to a species which at the moment we must refer to as *A.* (*S.*) *de-boeri* ssp. *de-meilloni* Edw., though final determination is still impossible, for the following reasons: In 1942 we found in Bwamba a *Stegomyia* sp., which was referable either to *A.* (*S.*) *de-boeri* ssp. *de-meilloni* or to *A.* (*S.*) *dendrophilus* Edw. The larvae resembled those of *dendrophilus*, but the male terminalia resembled those of *de-meilloni*. This species was found simultaneously, by Dr. P. C. C. Garnham and Mr. J. O. Harper, in Kenya. The material was studied by Mrs. van Someren (1946), who described the specimens in order to record their considerable range of individual variation. She concluded that perhaps we were dealing with a new racial form of *de-boeri*, but considered it inadvisable, at that stage, to form a final conclusion. Subsequently she obtained in Kenya further specimens of *de-meilloni*, in which the larva was typical (personal communication). The conclusion was thus that we were dealing with a new race or subspecies of *de-boeri*. In the meantime, however, we had examined the terminalia of the type male of *A. dendrophilus* at the British Museum. We found that Edwards had overlooked an important point—the presence in this species of two small spines on the basal plaque, previously believed to be diagnostic of *A. de-boeri* and *A. de-boeri* ssp. *de-meilloni*. The Bwamba mosquito thus resembles *A. dendrophilus* in the larval stage, and the male terminalia are exceedingly similar. The remaining differences are in adult coloration, and may be bridged by the great range of individual variation known to exist. Further, the type locality for *dendrophilus* is Oblogo, Gold Coast, so that we may be dealing with a new subspecies of *A. dendrophilus*. It is not possible to settle this matter finally, but it is here pointed out that it may subsequently be necessary to assign all Bwamba material so far recorded as *A. de-boeri* ssp. *de-meilloni* to *A. dendrophilus*, *A. dendrophilus* new subspecies, or even perhaps to a completely new species.

Virus was isolated three times from mixed lots of *Eretmapodites* ssp., as follows:

(*a*) From 441 mosquitoes, including 324 *E. chrysogaster* Graham group, 93 *E. inornatus* Newst. group, 22 *E. ferox* sp. n. (Haddow, 1946) and 2 *E. leucopus* ssp. *productus* Edw.

(*b*) From 942 mosquitoes, including 713 *E. chrysogaster* group, 106 *E. inornatus* group, 121 *E. ferox* and 2 *E. leucopus* ssp. *productus*.

(*c*) From 347 mosquitoes. The original lot totalled 482, including 367 *E. chrysogaster* group, 58 *E. inornatus* group, and 57 *E. ferox*, but 135 which were found dead on arrival at the laboratory were discarded without being reclassified.

It is necessary to discuss the two groupings used here. In Bwamba the *E. inornatus* group consists of approximately equal numbers of *E. inornatus* Newst. and *E. penicillatus* Edw. We have found (Haddow, 1936) that the only reliable method of distinguishing the females of these species is by examining the fore-claws under a high power of the microscope—a method which cannot be used while the mosquitoes are alive. The *E. chrysogaster* group is more complex.

We have found that the three common species in Bwamba are *E. chrysogaster* Graham, *E. semisimplicipes* Edw., and *E. grahami* Edw., and that the females cannot be reliably distinguished (Haddow, 1936). More recent studies by Mrs. van Someren have, however, revealed the presence of *E. intermedius* Edw. and four hitherto unknown species—easily identifiable by study of the male terminalia, but without reliable characters for the differentiation of females. Some, indeed, are still known only as males (van Someren, 1948). We are thus unable to say how many of the eight Bwamba species belonging to this group were included in the infected lots, nor can we hope to separate these species in future field or laboratory work of the same nature. In the case of the *E. inornatus* group we may safely assume that both species were present in all the infected lots, though we are unable to give the exact numbers of each.

The fact that the virus was isolated from relatively small lots of *Eretmapodites* spp. in three successive catches, that virus isolated from these mosquitoes had a short incubation in the initial passages in animals, and that it was readily recovered from Seitz filtrates of the mosquito suspensions, seems to indicate that these mosquitoes contained an abundance of virus and that, if the disease is insect-borne, this genus probably includes at least one vector. Though twice isolated from the *A. tarsalis* group, the virus was absent from the 6647 mosquitoes of this species in the second catch. Moreover, the failure to recover virus from Seitz-filtered suspensions of *A. tarsalis* group seems to indicate that these mosquitoes contained relatively little virus. The occurrence of the virus in *A. tarsalis* group mosquitoes may well have been casual, as they occur in vast numbers at Mongiro, forming by far the commonest group in the area. The failure of Seitz-filtered suspension of *A. de-boeri* ssp. *de-meilloni* lot 33 to yield virus indicates that these mosquitoes also probably contained little virus. Further, this species was taken in all three catches, yet only those of the second catch yielded virus. It therefore appears that this species may also be of less importance as a vector than one or other of the *Eretmapodites* spp.

Catches made at ground level and in the forest canopy at Mongiro and elsewhere, subsequent to the isolations of Rift Valley fever virus, indicate that each of the species concerned bites almost exclusively at ground level. This fact probably accounts for the absence of immunity among the wild monkeys of Bwamba.

Discussion.

In their studies on the transmission of Rift Valley fever, Daubney and Hudson (1933) noted that a lamb inoculated with a suspension of five wild-caught *Taeniorhynchus fuscopennatus* was immune to this disease after the inoculation. The virus was not isolated, but the inference is that the mosquitoes were infected. These authors also showed that sheep which are protected from mosquito bites are likewise prevented from contracting the disease under natural conditions, and that protection from mosquitoes at night alone reduced the hazard of infection significantly.

The six isolations of virus here reported from mosquitoes caught in a relatively circumscribed area over a period of 39 days is evidence enough of virus activity of epidemic proportions. However, the outbreak with which we are here concerned is very different from outbreaks studied by the workers in Kenya. Whereas the outbreaks in Kenya involved humans, sheep and cattle, the outbreak in

the Semliki Forest at Mongiro could not have involved any of these, as the area is uninhabited. It must have involved wild animals, yet the only suggestion of evidence obtained on this point is the observance of a dead buffalo and a sick buffalo calf in the catching area at the time. Immunity surveys of the mosquito catchers and of residents of the nearby Semliki Plains were negative, as were surveys carried out in wild monkeys of the Bwamba lowlands. Daubney and Hudson (1933) found the buffalo to be susceptible to experimental inoculation with Rift Valley fever virus. These animals are numerous in the Mongiro area, as are buck of several species, giant forest hogs and Uganda red hogs, elephants and other wild animals. It seems probable that we were dealing with the mosquito phase of an epizootic " in the wild " involving animals of species which remain unknown

The isolation of a virus from mosquitoes is not positive proof that those insects are actively involved in cycles of transmission. Indeed, in the experience at Mongiro, it seems unlikely that *Aëdes de-boeri* and *Aëdes tarsalis* were vectors, since neither of these species was infected on every occasion, and the evidence indicates that neither contained virus in large quantity. The situation was rather different, however, with reference to the *Eretmapodites* spp. These mosquitoes contained Rift Valley fever virus in each of three successive 5-day catches made during the 39-day period, and the indications were that they contained the virus in relatively large amounts in each instance. We assumed, therefore, that if the mosquitoes implicated included a vector species, it must be one of the genus *Eretmapodites* included in the catches. The soundness of this assumption was borne out by the successful experimental transmission of the infection with *E. chrysogaster* group, as will be shown in a separate communication (Smithburn and Haddow, in the press).

SUMMARY.

A filterable virus with hepatotropic affinities, shown by immunological methods to be Rift Valley fever virus, was isolated from six different lots of mosquitoes caught in the uninhabited Semliki Forest in western Uganda in 1944. The mosquitoes involved included at least six species of the genus *Eretmapodites* and three of the genus *Aëdes*. Indications were that the latter may have been only casually infected, and that the local vector, if any was included in the species implicated, was one of the genus *Eretmapodites*.

Epidemiological studies pertaining to the infection are reported from which it is concluded that the outbreak did not involve humans, but probably affected some unidentified species of wild animals.

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BODILY REACTIONS TO TRAUMA. THE EFFECT OF ISCHAEMIA ON MUSCLE PROTEIN.

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OUR previous work on the effect of ischaemia on the thromboplastic activity of extracts prepared from muscle (Stoner and Green, 1947) led us to consider the possible changes in the muscle proteins which might be produced by that form of trauma. In the previous paper we discussed the possibility that the increased thromboplastic activity of the ischaemic muscle extracts might be due to the liberation of the active material from an intracellular store of inactive "combined thromboplastin" possibly as a result of the action of proteolytic enzymes. Cullumbine and Rydon (1946) have suggested a similar mechanism for the liberation of leukotaxine. Subsequent work on the thromboplastic activity of muscle extracts has provided little, if any, evidence in favour of such an hypothesis, but led us to a study of proteolysis in ischaemic muscle. Special attention has been paid to possible differences between ischaemic and autolytic muscle since previous work (Stoner and Green, 1947) has shown that their behaviour is not always the same.

It was first necessary to find the solubility of the muscle proteins in salt solution, and the method described by Deuticke (1930, 1932) was used. In confirmation of the results of Erdós (1943), the decrease in the solubility of muscle proteins after a period of 5 hours autolysis was found to be very slight. The same period of ischaemia produced a similar slight change in solubility.

Maver, Johnson and Voegtlin (1935) have shown that under conditions of low oxygen tension and acid reaction, i.e. under conditions which will be present in ischaemic and autolytic muscle, proteolysis occurs. Our first step in investigating this possibility was to determine the acid soluble nitrogen content of the muscle after periods of autolysis and ischaemia, since Bradley (1938) considers this test to be the best indication of primary cleavage of protein. However, no changes were found during the short periods studied, and it was felt that this method was too crude for our purpose. Accordingly we determined the amino acid content of the muscle with the results to be described.

METHODS.

Albino rats from one source were used throughout these experiments, and muscle ischaemia was produced by the metal clamp usually used in this department applied to the hind limb under ether narcosis. The general effects of muscle ischaemia produced in this way have been described previously (Green, 1943). The period of ischaemia most frequently used in these experiments was 4–5 hours. Bilateral hind limb ischaemia of this duration is usually fatal in the rat. The present experiments showed that ischaemia of this type is not complete.

In experiments on autolytic muscle the rats were killed and the muscles of one hind limb sampled immediately. The animal then remained in the same environmental conditions as the " clamped " rats for varying intervals of time, after which the muscles of the other hind limb were sampled.

The muscle samples (gastrocnemius and adductores) were extracted as quickly as possible after removal by grinding them up with quartz and 10 ml. of 10 per cent trichloroacetic acid per g. fresh tissue. These extracts were filtered twice through Whatman No. 42 filter-paper and stored at 0° C. The combined tyrosine and tryptophane content of these extracts was determined by comparing the colour developed with the phenol reagent of Folin and Ciocalteu (1927) with that developed by a standard solution of tyrosine. The results are expressed in terms of tyrosine. In a few experiments the α-amino nitrogen content of these extracts and of extracts prepared according to the directions of Russell and Long (1946) was determined by the method of Frame, Russell and Wilhelmi (1943).

The α-amino nitrogen content of the blood was determined by the method of Frame, Russell and Wilhelmi (1943) as modified by Russell (1944). The blood for this determination was obtained when possible from the tail or otherwise by cardiac puncture.

The muscle temperature was determined by inserting a copper-constanton thermocouple into the muscle through a skin puncture, the other end of the thermocouple being kept at 0° C. and the temperature difference being recorded by a mirror galvanometer.

The pH of the muscle was determined by a method similar to that of Kahler and Robertson (1943), using a Marconi electrometer and a glass electrode with a bulb 7 mm. in diameter. During the recording of the pH the muscle area was sprayed with a stream of 5 per cent carbon dioxide in oxygen. The rats were kept under light ether anaesthesia during the determination.

RESULTS.

Proteolysis in ischaemic and autolytic muscle.

Control experiments showed (Table I) that although the absolute level of the amino-acid content of normal muscle varied from animal to animal, the amounts present in the muscle of the two hind limbs of an animal were approximately the same. The variations in the absolute levels may reflect differences in the environmental conditions at the time of the experiment. Tabor and Rosenthal (1947) have shown the importance of the air temperature in determining the outcome of experiments on limb ischaemia. As it was not possible to control the environmental temperature absolutely, in each table the number of the

experiment is given so that it is possible to see which animals were used at the same time under the same environmental conditions.

TABLE I.—*Tyrosine Content of Muscle from the Hind Limbs of Normal Rats.*

Exp. No.	Tyrosine content (mg. per g. dry wt.). Right leg.	Left leg.
102	0·37	0·36
102	0·40	0·35
102	0·36	0·38
140	0·17	0·19
141	0·24	0·24
141	0·24	0·23
147	0·18	0·18
157	0·23	0·26
158	0·22	0·23

Mean difference between values for right and left leg = 6·3 per cent.

Despite such slight changes as may have occurred in the environmental conditions between experiments, the constancy of the tyrosine content of the muscle from the two hind limbs makes it possible to use one limb as the control for the other. When the tyrosine content of ischaemic muscle was compared with that of the opposite normal muscle it was found (Table II) that a large increase occurred in the muscle during a 5-hour period of ischaemia. The rate of increase was greatest during the first 2 hours, after which it declined somewhat, accelerating again during the last hour of the 5-hour ischaemic period. The tyrosine content of muscle from the unaffected limb remained within the normal range shown in Table I. From this standpoint the control muscle may be considered normal, although this is not always the case, e.g. nucleotide content (unpublished results).

TABLE II.—*Effect of Different Periods of Ischaemia and Autolysis on the Tyrosine Content of Rat Muscle.*

Tyrosine content (mg. per g. dry weight.)

2 hr. ischaemia.			4 hr. ischaemia.			5 hr. ischaemia.		
Exp. No.	Normal.	Ischaemic.	Exp. No.	Normal.	Ischaemic.	Exp. No.	Normal.	Ischaemic.
173	0·22	0·30	140	0·13	0·23	106	0·28	0·56
174	0·22	0·29	141	0·23	0·44	106	0·32	0·53
174	0·14	0·27	142	0·22	0·36	105	0·21	0·44
175	0·19	0·29	144	0·16	0·39	105	0·25	0·45
176	0·18	0·29	155	0·25	0·49	103	0·35	0·63
			155	0·27	0·48	103	0·28	0·56
	Aver. increase 55 per cent.			Aver. increase 81 per cent.		175	0·22	0·51
						177	0·26	0·60
						177	0·23	0·60
						178	0·20	0·74
							Aver. increase 123 per cent.	

TABLE II—*continued.*

2 hr. autolysis.			5 hr. autolysis			24 hr. autolysis.		
Exp. No.	Normal.	Autolytic.	Exp. No.	Normal.	Autolytic.	Exp. No.	Normal.	Autolytic.
173	0·26	0·30	106	0·23	0·37	111	0·25	0·80
174	0·22	0·32	106	0·26	0·32	111	0·25	0·88
174	0·18	0·25	105	0·22	0·26	107	0·37	0·56
175	0·14	0·30	105	0·32	0·29	107	0·23	1·22
176	0·26	0·27	103	0·41	0·52	179	0·31	1·33
			103	0·52	0·44	179	0·30	1·28
			175	0·23	0·40	179	0·35	1·40
			177	0·24	0·47			
			177	0·22	0·36			
			178	0·29	0·52			
	Aver. increase 48 per cent.			Aver. increase 44 per cent.			Aver. increase 267 per cent.	

In autolysing muscle the amount of proteolysis which occurred during the first 2 hours after the death of the animal was not significantly different from that which occurred during the same period of ischaemia (Table II). However, after the first 2 hours, protein breakdown, as assessed by the increase in tyrosine, proceeded much more slowly in the autolysing muscle. After 5 hours its tyrosine content was very much less than that of muscle after 5 hours ischaemia. The amount of tyrosine found after 24 hours suggests that after the first 2 hours proteolysis proceeds at a fairly steady rate.

The experiments in which the α-amino nitrogen content of the extracts was determined also showed that proteolysis was occurring more rapidly in the ischaemic muscle.

Effect of restoration of the circulation.

The changes in the tyrosine content of the muscle were also followed after removal of the clamp and restoration of the circulation to the damaged part. These changes are shown in Fig. 1. Shortly after the release of the constriction the water content of the damaged muscle rose and remained high during the 7-day period studied. The tyrosine content per g. dry muscle rose sharply during the first hour after release of the clamp. It then fell during the next 2 days, to be followed by a secondary rise, which continued progressively until the end of the 7-day experimental period. The tyrosine content of the opposite undamaged muscle remained within the normal range throughout this period.

The α-amino nitrogen content of the damaged muscle at the end of the 7-day period was 47 per cent higher than that of the opposite normal side. (Normal variation between the muscle of the two limbs $\pm$ 10 per cent.)

The expression of these results in terms of dry weight of the muscle may introduce a possible error, since the fluid which passes into the muscle is not water, but an exudate of plasma containing aminoacids and proteins. Although these latter may not enter the muscle cell they will lie in the tissue spaces, where they may be broken down by proteolytic enzymes which have become extracellular as a result of injury (Zamecnik, Aub, Brues, Kety, Nathanson, Nutt and

Pope, 1945), and their degradation products will be included in the estimation. For this reason we have included in Fig. 1 the curve obtained by calculating the results in terms of the wet weight of the muscle. Both curves should be considered in assessing the true course of events. It would then seem that proteolysis continues in the muscle for about 2 hours after the restoration of the

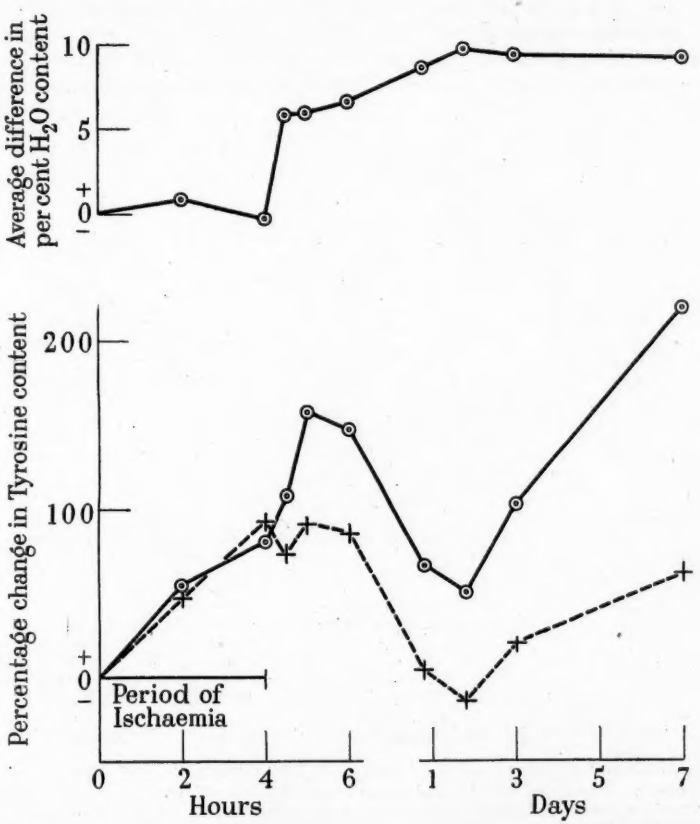

FIG. 1.—The lower curves show the average percentage difference between the tyrosine content of the muscle of the hind limb of a rat during and after a 4-hr. period of ischaemia and that of the opposite normal muscle. Data calculated in terms of the dry weight of the muscle for curve ⊙——⊙ and in terms of the wet weight for curve ×– – – – ×. The upper curve shows the average difference in the water content of the damaged and normal muscle.

circulation. During the next 2 days the products of this proteolysis disappear, either as a result of further degradation *in situ* or by removal into the blood stream. By the 3rd day the tyrosine content of the post-ischaemic muscle is again increasing, and continues to do so over the period studied.

Muscle temperature during ischaemia and autolysis.

After the death of the animal the temperature of the muscle fell rapidly during the first 2 hours (Fig. 2), by which time it had almost reached room temperature. It continued to decline slowly until room temperature was reached at about 5 hours. The temperature of ischaemic muscle also fell rapidly during the first 1–2 hours, after which a very gradual rise occurred. Thus for the greater part of the ischaemic period the temperature of the ischaemic muscle was about 5° C. higher than that of corresponding autolytic muscle (Fig. 2). This temperature difference may be due either to heat generated by chemical reactions

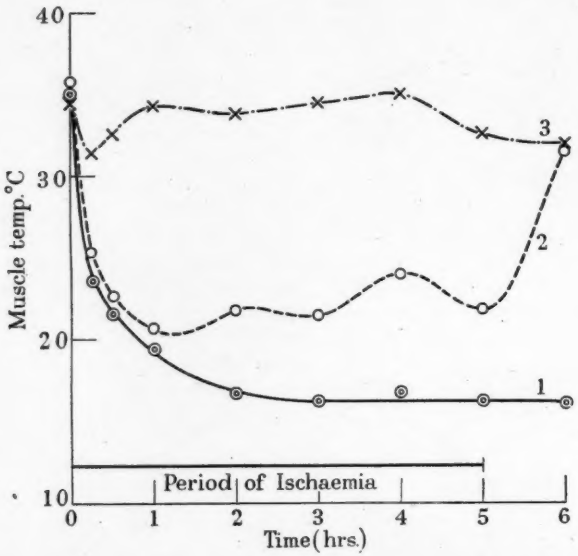

FIG. 2.—Effect of autolysis and ischaemia on the temperature of the muscle of the hind limb of the rat.
Curve 1: Rat killed at zero time and muscle allowed to autolyse *in situ*.
Curve 2: Clamp applied to left hind limb of a rat at zero time.
Curve 3: Normal (right) hind limb of the same rat.
Room temperature = 14°C.

taking place in the ischaemic but not in the autolytic muscle, or to a feeble return of circulation through anastomatic channels in bone. The latter seems the more probable explanation in view of the size of the temperature difference.

The rapid return of the temperature to normal on release of the clamp indicates that our method of producing ischaemia does not cause permanent damage to the circulation. This was confirmed in experiments in which large doses (10 M.L.D.) of strychnine nitrate were injected into the limb whilst the clamp was still in position. No serious symptoms occurred until the clamp was removed, when death in convulsions occurred in a few minutes.

Muscle pH during ischaemia and autolysis.

Previous attempts (Rous and Drury, 1928 ; Billings and Maegraith, 1937) to determine the reaction of ischaemic tissues have been made with intravital indicators. This method, which, at the best, can only give an approximation to the pH of the tissue, was found to be unsuitable for our experiments where the dark colour of the muscle, especially after applying the clamp, concealed any changes which might occur in the colour of the indicator. We hoped to obtain a closer approximation to the actual pH occurring in the muscle by using a glass electrode system.

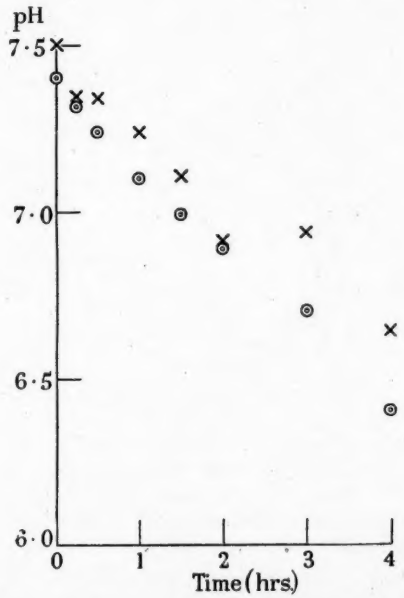

FIG. 3.—Effect of autolysis and ischaemia on the pH of the muscles of the hind limb of the rat.
⊙ = values obtained from left leg of a rat which was killed at zero time and muscle allowed to autolyse *in situ*. pH of muscles of right leg at the end of 4 hr. = 6·40.
× = values obtained from right leg of a rat in which both legs were clamped at zero time. pH of muscles of left leg at end of 4 hr. = 6·80.

After the death of the animal the pH of the muscle fell steadily, reaching a value of 6·2–6·6 in 4 hours. The pH of ischaemic muscle also decreased over the 4-hour period. The fall tended to be rather irregular, but the final value was in the same range as that of autolytic muscle (Fig. 3).

On restoration of the circulation to the ischaemic limb the pH of the muscle rose rapidly at first and then more slowly, reaching the normal range within about 2 hours (Fig. 4). Determinations of the muscle pH over the remainder of the 7-day period (Table III) showed that the pH on the damaged side was always slightly less than on the normal side.

TABLE III.—*pH of Rat Hind Limb Muscle taken at Intervals after a 4-hour period of Ischaemia.*

Time (hr.) after restoration of circulation.	No. of rats.	Muscle pH.	
		Normal limb.	Post-ischaemic limb.
20	5	7·42	7·30
44	4	7·36	7·30
72	5	7·41	7·22
168	4	7·35	7·17

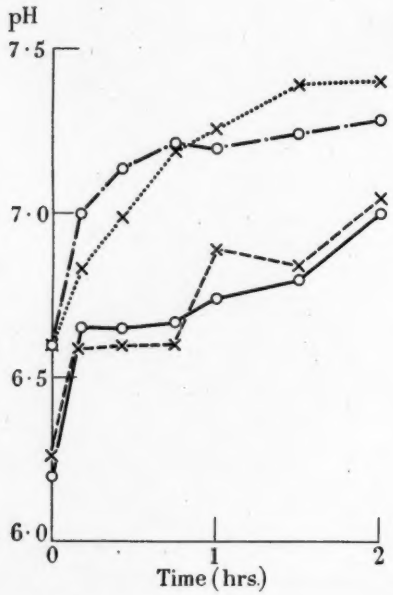

FIG. 4.—Effect of restoration of the circulation to the hind limb of a rat after 4 hr. ischaemia on the pH of the muscles. Each curve represents the results obtained in an individual rat. pH of muscle of normal leg at end of experiment:

Curve O——•——O; 7·46.
" ×– – – – – –×; 7·36.
" ×· · · · · · ·×; 7·40.
" O————O; 7·44.

Effect of limb ischaemia on the amino-nitrogen level of the blood.

It is known that after limb ischaemia, as after other forms of injury, there is an increase in the amino-acid content of the blood (Hoar and Haist, 1944). Our object was to find if there was any relationship between this increase and the fall in the tyrosine content of the muscle between 2 and 44 hours after the injury. Naturally, with ischaemia of only one hind limb large increases in the α-amino nitrogen content of the blood after release of the clamp were not to be expected,

but the significance of the changes which occurred was enhanced by the fact that the level in similarly bled controls fell over the course of the experiment. It was found (Table IV) that the α-amino nitrogen level of the blood in the experimental animals did not fall after removal of the clamp, and in the majority of rats was raised during the period from 2 to 44 hours after the restoration of the circulation.

TABLE IV.—*The Effect of Ischaemia of One Hind Limb on the α-Amino Nitrogen Content of the Blood of the Rat.*

Blood amino N. content (mg. per 100 ml.)

Exp. No.	Rat No.	Treatment.	Time when specimen taken.				
			Before ischaemia.	Before removal of clamp.	2 hr. after removal.	20 hr. after removal.	44 hr. after removal.
197	1	4 hr. ischaemia : 1 hind limb	11·4	8·9	12·3	9·1	12·1
197	2	nil	9·2	—	9·8	7·4	—
197	3	nil	12·2	12·6	9·4	—	—
197	4	4 hr. ischaemia : 1 hind limb	8·8	8·5	9·4	15·8	—
204	2	4 hr. ischaemia : 1 hind limb	11·6	12·3	15·0	12·1	12·9
204	3	4 hr. ischaemia : 1 hind limb	12·8	—	16·6	14·0	—
204	4	nil	12·5	13·1	12·6	13·5	11·8

DISCUSSION.

Our results show that proteolysis, as judged by an increase in amino acid content, occurs in muscle deprived of the major part of its blood supply. This was to be expected, but it was interesting to observe that the amount of proteolysis was much greater in ischaemic than in autolysing muscle. This finding was in agreement with other results, showing that the changes in ischaemic muscle are not identical with those in autolysing muscle (Stoner and Green, 1947; unpublished results). In the case of the changes in the thromboplastic activity of ischaemic muscle the difference was one of kind; here it would appear to be one of degree. In seeking an explanation for this difference in the amount of proteolysis we considered the changes in the pH and temperature of ischaemic and autolysing muscle. Under our experimental conditions the pH changes in the two types of muscle were very similar, but the temperature of the ischaemic muscle after the first 1–2 hours of the experiment was considerably higher than that of the corresponding autolysing muscle. The greater amount of protein breakdown in the ischaemic muscle may be due to its higher temperature. This explanation receives support from the similar degree of proteolysis in both ischaemic and autolytic muscle during the first 2 hours of the experiment, during which temperature conditions are approximately the same.

Incidentally it would seem from the experiments on muscle temperature that absolute ischaemia, whilst preserving the integrity of the limb and vessels, is difficult or impossible to produce, even though, as in these experiments, the degree of occlusion is sufficient to prevent effective amounts of strychnine from leaving the limb.

The other interest of our results lies in the changes occurring in the muscle after restoration of the circulation. For the first hour after removal of the clamp proteolysis occurs at an even greater rate than during the period of ischaemia. This is probably explained by the increase in the temperature of the muscle after the release of the clamp whilst the pH is still in the acid range. When the pH of the damaged muscle approaches the normal value this phase of proteolysis stops and the amino-acid content of the muscle falls. These experiments indicate that proteolysis of this type, i.e. that due to the intracellular cathepsins, is determined by the pH level in the tissues. This was shown *in vitro* by Maver *et al.* (1935). Anoxia is not the most important factor; if it were then more proteolysis should occur in the autolytic tissue where the degree of anoxia is greater, whereas the reverse was found.

As already pointed out, there are two possible explanations for the decrease in the amino-acid content of the muscle during the period from 2 to 44 hours after release of the clamp. The excess amino-acid may undergo degradation *in situ*, or it may be removed into the blood stream. The experiments with strychnine show that there is adequate absorption from the damaged limb after release of the clamp. Obviously the possibility of breakdown *in situ* cannot be excluded, but the small increases observed in the α-amino nitrogen level of the blood during this period suggest that at least part of the excess enters the blood stream. Hoar and Haist (1944) postulated that the increase in the α-amino nitrogen level of the blood in ischaemic muscle shock is largely due to processes occurring in the damaged tissue. Our results complement theirs by showing the nature of the changes giving rise to an excess of amino-acid in the muscle, which are seen to be similar to those occurring in the muscle of rats in haemorrhagic shock (Russell and Long, 1946). According to Long and his co-workers (Russell, Long and Engel, 1944; Wilhelmi, Russell, Engel and Long, 1945) the liver of the animal in shock is unable to metabolize amino-acids to the same degree as that of the normal animal, and this factor plays a part in raising the blood level of these acids. It would be expected that relatively little liver damage would occur in our experiments, where only a sub-lethal degree of trauma was used, and this may account for the small increases in the blood level observed.

Whilst proteolysis occurring during and shortly after the period of ischaemia is most easily attributed to the action of the tissue cathepsins, these enzymes are probably not responsible for the second increase in the amino acid content of the muscle between 3 and 7 days after release. It is unlikely, because the pH of the muscle at this time is more favourable for protein synthesis (Maver *et al.*, 1935), and far above the optimum range for the tissue cathepsins described by Bergmann (1942). Barnes and Trueta (1942) have shown, in the rabbit, that on restoration of the circulation to an ischaemic limb the muscles become infiltrated with cells. At first these are polymorphonuclear leucocytes, but after 72 hours large numbers of large mononuclear cells appear. The same sequence of events was found to occur in the rat. The reaction of the tissue throughout this period is slightly alkaline, and this observation therefore supports the view of Steinberg and Dietz (1938) that no relationship exists between the pH of the exudate and its cytological picture (Menkin, 1934). According to Opie (1922), proteolytic enzymes were first demonstrated in polymorphonuclear leucocytes by Muller in 1888. Barnes (1940) has shown that the enzyme present in these cells in the cat is active in the normal pH range, and we have confirmed

this for the rat. Opie showed that the large mononuclear cells also contained a proteolytic enzyme most active in a slightly acid medium. It is probable that the enzymes contained in these cells, especially the large mononuclear ones, are responsible for the protein breakdown which occurs during the period of 3–7 days after the ischaemia. The pH conditions in the limb may not be optimal for the activity of the enzyme of the large mononuclear cells, but digestion may proceed inside or on the surface of these cells, and our method of determining pH does not enable us to assess the local pH conditions at these sites. The substrate for this enzyme, though presumably largely damaged muscle, may partly consist of proteins in the inflammatory exudate.

It is not known what relationship, if any, exists between this breakdown of tissue protein and the nitrogen lost in the urine during the "post-shock metabolic response" described by Cuthbertson (1942). The time of onset of the two phenomena is similar. Those who have studied the nitrogen loss after injury are agreed that the amount of nitrogen lost cannot be entirely accounted for by local changes. Nevertheless the protein breakdown which we have shown to occur in the damaged tissue during this period must contribute to it in some degree.

SUMMARY.

The breakdown of protein in rat muscle during autolysis and during and after ischaemia of a type known to produce fatal shock has been followed by determining the amino-acid content of the muscle. Two types of proteolysis were found to occur:

In autolytic muscle and muscle during, and shortly after, ischaemia proteolysis is due to the activity of tissue cathepsins. In experiments lasting 2 hours the amount of proteolysis which occurred in autolytic and ischaemic muscle was the same, but in longer experiments much more proteolysis occurred in the latter, and this was thought to be due to the higher temperature of the ischaemic muscle. The pH fell equally in both types of muscle. Restoration of the circulation to the damaged limb was followed by an immediate outburst of proteolytic activity, thought to be due to the further increase in temperature whilst the pH was still low. The main factor in determining the onset of this type of proteolysis would appear to be the pH of the tissues. Part at least of the products of this protein breakdown are removed by the blood stream.

The second phase of protein breakdown does not commence until about 3 days after the period of ischaemia and continues at least for the next 4 days. The pH conditions at this time are not suitable for the action of the tissue cathepsins, and the enzymes producing this proteolysis are probably derived from the leucocytes which infiltrate the damaged muscle.

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INFLUENZA A IN GREAT BRITAIN 1946–47.

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THE last major influenza epidemic in Britain occurred in the autumn of 1943 (Andrewes and Glover, 1944) and was caused by the influenza A virus. During the winter of 1945–46 influenza B was widespread throughout this country (Dudgeon, Stuart-Harris, Glover, Andrewes and Bradley, 1946), and it was therefore possible, on epidemiological grounds, that an influenza A epidemic might occur during the winter 1946–47. By the end of December, 1946, no influenza had been reported in this country, but by the second week in January cases of influenza began to appear, and during the ensuing weeks many typical localized outbreaks were seen.

This account deals with the laboratory investigations of several of these outbreaks ; these were undertaken partly with a view to assessing the comparative merits of inoculation into ferrets and the amniotic cavities of fertile hen's eggs as a means of virus isolation and typing. Routine serological tests (Hirst tests)

were also carried out as an aid to the diagnosis of influenza throughout the country as a whole, and in particular in those communities in which a trial of influenza virus vaccine was being carried out. A report on the results of these trials is being published elsewhere (Mellanby, Andrewes, Dudgeon and Mackay, 1948).

GENERAL EPIDEMIOLOGY.

The first indication of influenza came from prisoner-of-war camps in the Midlands, where an explosive and extensive epidemic occurred during the second week in January. A few days later several cases were seen in the wards of a London hospital, though no epidemic developed. From the end of January until the middle of March many localized epidemics were seen in preparatory and public schools throughout the country. The majority of these epidemics were mild, varying only in the attack-rate ; the average was about 25 per cent, but in one case the incidence reached 70 per cent. In many instances these school epidemics were followed by a high incidence of streptococcal pharyngitis and measles. In the army units investigated, the incidence of influenza was not as high as in the school populations. At one unit in Northern Ireland, however, a relatively extensive epidemic started in the second week in January, and involved some 26 per cent of 1500 new recruits but only 1·2 per cent of the 250 permanent staff. No general epidemic developed amongst the civilian population, although there was an increased prevalence of influenza during the first two months of the year. The number of deaths in the 126 Great Towns, as notified by the Registrar-General, showed a peak of 211 during the first week in February ; this was less than the corresponding figure for 1946 (304). There was also no undue absenteeism in industry, and reports from the Continent indicated that there was very little upper respiratory infection amongst British Service units or amongst the civilian population in Germany.

In general, therefore, there were widespread but localized epidemics throughout the country, with a relatively high incidence on the younger age-groups, as was the case in 1946.

Laboratory Investigations.

I. *Virus isolation in chick embryo.*

The method of virus isolation by amniotic inoculation originally described by Burnet in 1940 has, with technical improvements, become an extremely sensitive method, not only for primary isolation, but for the rapid identification of the antigenic type of virus (Beveridge and Burnet, 1946). Hirst (1945) showed in a review of some 56 cases that the amniotic method was the most sensitive for primary isolation. Previous work in this country has, however, not been able to confirm these findings (Andrewes, Glover, Hudson, Lush and Stuart-Harris, 1941 ; Stuart-Harris, Glover and Mills, 1943 ; Andrewes and Glover, 1944). During the winter 1945–46 several influenza B strains were isolated by the amniotic route (Dudgeon *et al.*, 1946). This better success may have been due to the fact that throat washings were not filtered through a gradocol membrane, but were inoculated direct into the amniotic cavity with the addition of antibacterial agents. It is doubtful whether the earlier lack of success was due to a combination of several technical factors, or to differences between the viruses.

In view of these previous difficulties it was decided to examine throat washings during the season 1946–47 in numbers comparable with those examined in 1945–46, in an attempt to assess the factors determining successful virus isolation by amniotic inoculation. Throat washings were taken from typical cases of influenza in the acute stage of the disease. The patient was asked to gargle 15 c.c. of saline, to which was added 5 c.c. 10 per cent horse serum broth. The washings were kept at ice-box temperature until ready for inoculation. If not used within 48 hours of collection, the washing was stored at −76° C. Washings were centrifuged at 2500 r.p.m. for twenty minutes in order to sediment as much mucus and debris as possible. Sufficient penicillin was added to produce a final concentration of 150 units per c.c. Each washing was inoculated into a batch of 5 eggs previously incubated for 13 days at 37–38° C.; each egg received, immediately prior to inoculation, 0·1 c.c. 5 per cent sodium sulphamerazine into the amniotic cavity and then 0·25 c.c. of the washings and penicillin. Eggs were then incubated at 35·5–36° C. for a further 4 days after inoculation. With few exceptions, the penicillin and sodium sulphamerazine produced sufficient bacteriostasis, and additional doses of penicillin were not necessary. After 4 days' incubation all live embryos were placed in the ice-box for 1 hour to diminish liability to haemorrhage when they were opened; the amniotic fluids were then withdrawn and tested with guinea-pig and fowl red cells for the presence of haemagglutination.

We used Salk's modification of Hirst's original methods throughout this investigation (Salk, 1944). In this method a weaker concentration of red cells (0·25 per cent) is employed, and the end point is determined by observing the pattern of agglutinated cells on the bottom of the tube. In Hirst's method 2 per cent red cells are used, and the end point in the titration is determined by the clearing of the supernatant fluid matched against a standard red cell suspension. Salk's method tends to give slightly higher haemagglutinin values. In testing these fluids 0·25 per cent red cells were added to a 1/10 dilution of amniotic fluid and the resulting agglutination noted. Selected fluids which had shown a positive agglutination by this method were titrated with the red cells of both species in order to determine the relative titres against fowl and guinea-pig cells (F/G titres) (Burnet and Stone, 1945).

With few exceptions there was little change in the macroscopic appearance of the embryo, lungs or membranes. The only change which was at all noticeable was a turbid appearance of the tracheal and amniotic fluids in many, but not all, embryos. Tracheal fluids from infected embryos showed a high cellular content on microscopic examination.

Fifty-five throat washings were tested by this method, and virus was isolated from 32 (60 per cent) different cases on primary passage. Negative garglings which received a second or third amniotic passage did not in a single case become positive. Similarly, negative amniotic fluids from a group of eggs in which some were positive also failed on further passage to produce any sign of infection. Table I shows a comparison between the washings tested this year and those tested during the 1945–46 B epidemic. The symptoms produced in the ferrets were not very striking, but these results indicate the relative sensitiveness of the amniotic method for virus isolation. The antibody response to the homologous virus in each case was better than against the standard PR8 or LEE strains.

TABLE I.—*Virus Isolation during Two Influenza Outbreaks.*

Year.	No. tested.	No. isolated in eggs.	No. tested in ferrets.	Response in ferrets.				
				Pyrexia.	Antibody response.			
					PR8	LEE	'46 (B)	'47 (A)
1945–46	54 (B)	12	47	2	—	4/47	8/47	—
1946–47	55 (A)	32	16	5	6/16	—	—	13/16

Throat washings on primary passage only infected a proportion of the embryos, but on subsequent passage with a positive fluid all embryos became infected. In 5 cases only 1 egg out of each group of 5, and in a further 8, only 2, showed agglutination, but in the majority of cases 3 or 4 eggs in each group were infected by the fourth day. These results confirm the findings of other workers that 5 eggs in each group is an absolute minimum for diagnotic tests ; 6 eggs is probably a more satisfactory number for use.

In all cases virus on primary isolation was in the " O " (" original ") phase or intermediate (O–D) phase, as shown by the higher agglutinin titre with guinea-pig cells than with fowl cells (Burnet and Stone, 1945). Some fluids showed a typical " O " phase reaction as shown by BAR (1) in Table II and others a partial change from O —→ D, which we have called intermediate or O–D. We did not distinguish Burnet and Stone's ω and δ phases. The fact that amniotic fluids contained virus in the " O " phase indicated that current strains were A viruses, since an O-phase has not yet been shown for virus B. When however these fluids were tested with stock immune A (PR8) serum for specific inhibition no marked degree of agglutinin-inhibition was obtained. It was considered at the time that these strains, although related to the PR8 virus, might differ substantially from it in their antigenic structure, and further work was planned to investigate this problem. On further passage a higher agglutinin titre with fowl red cells was produced, as is characteristic of the typical " D " (Derivative) phase.

TABLE II.—*To Show the Relative Fowl/Guinea-pig Titres of Amniotic Fluids on 1st and 2nd passage.*

Throat washing.	F/G titres of individual embryos.					Phase.
	1	2	3	4	5	
BAR (1) .	0/10	0/20	0/160	40/320	20/320 .	O
EVA (2) .	80/320	0/80	80/320	20/160	20/160 .	O–D
BAR (2) .	320/160	320/160	160/80	160/80	320/120 .	D

Note.—The numbers in brackets after the strain refer to the number of amniotic passages the virus has undergone.

By using limiting dilutions, as suggested by Burnet and Stone (1945), the virus could be maintained in the O-phase for several passages. All strains were readily adapted to growth in the allantoic cavity, producing fluids with a good haem-agglutinin titre. Several of these were used for ferret inoculation and the production of specific immune serum for antigenic analysis.

II. *Virus isolation in ferrets.*

Throat washings from 16 cases were inoculated intranasally into anaesthetized ferrets. In every instance the specimen had been stored at −76° C. for a short period. In five animals there was a significant rise in temperature but symptoms were very mild ; in the remaining animals there was no febrile reaction, no clear-cut symptoms of coryza or nasal obstruction, and little change in the turbinates or respiratory tract of such animals as were killed. In general, the mildness of the reaction was reminiscent of the results obtained in ferrets during the influenza B epidemic of 1945–46. Nevertheless samples of serum of such ferrets usually showed a significant rise in antibody to the PR8 virus, and a still greater rise to the homologous virus.

In addition 9 ferrets were inoculated with allantoic fluid infected with strains of virus corresponding in some cases to material inoculated in the form of garglings. There was a sharp febrile reaction and a moderate to slight reaction in 6, whilst 3 were negative. Strain NEW, however, as a result of passage through 5 generations, became progressively more virulent, and at the 2nd pass behaved like a typical A strain ; the febrile reaction was more acute and symptoms were more clear-cut. Two further strains, WIL and BAR, also showed enhancement of virulence on passage. An unsuccessful attempt was made to adapt strain NEW to mouse lung, and passage was discontinued after the 3rd pass. Except in these instances no attempt was made to establish the strains by serial passage in ferrets.

Cross-immunity tests carried out on 15 ferrets indicated that animals recovered from infection three weeks earlier with PR8 or with either of the 1947 strains BAR or NEW were immune to any of the others. An exception was a ferret infected with BAR which reacted with a fever of 104·8° F. when inoculated with NEW 14 days later.

III. *Adaptation to mice.*

Several of the strains isolated in the chick embryo were selected for adaptation to mouse lung. Four strains, BAR, EVA, WIL, JAC, one each from typical outbreaks, and each having had two amniotic and two allantoic passages, were used for this purpose.

Groups of 6 mice (Parkes strain) were inoculated intranasally with undiluted allantoic fluid. Half of the group were killed on the 3rd day for passage to a further 6 mice and the remainder were killed on the 6th day ; the degree of pulmonary consolidation was noted. In view of the difficulties experienced in 1945–46 in adapting B strains to mice, it was decided to pass the mouse lung material back to eggs on each mouse passage so that, if specific lesions were absent, presence of the virus could be readily detected by haemagglutination.

The four strains behaved in very much the same manner. Visible pulmonary lesions were not evident until the 4th pass, and then only very small lesions involving 1/8 of the lung or less were seen. Material, however, when inoculated into eggs allantoically (10^{-2} dilution) produced haemagglutination. On further passage the degree of consolidation increased until the 12th passage, by which time the strains were killing mice regularly with total pulmonary consolidation.

Previous attempts to adapt A strains to mice have produced pulmonary lesions more readily. Stuart-Harris, Andrewes, Smith and others (1937) and

Burnet, Beveridge, Bull and Clark (1942) had little trouble in adapting their A strains during extensive epidemics. Andrewes and Glover (1944) however were only able to adapt 2/9 strains during an extensive A epidemic in the autumn of 1943, and attempts to adapt the B strains to mice in 1946 were altogether unsuccessful (Dudgeon *et al.*, 1946).

IV. *Serological investigations.*

Samples of serum for the serological diagnosis of influenza were collected from many outbreaks and from sporadic cases throughout the country. A few sera were received from the Rhine Army, but little respiratory infection was encountered in Germany during the first three months of 1947.

The method used for the agglutinin-inhibition test was Salk's (1944) modification of Hirst's original method. Serum samples taken in the acute and convalescent stage of the disease were inactivated and stored at ice-box temperature until ready for use. The antigen used in these tests was dried allantoic fluid kindly sent in 1943 by the Rockefeller Foundation ; this was reconstituted in saline. The standard dose of antigen used in these tests consisted of $2\frac{1}{2}$ Minimal Agglutinating Doses (M.A.D.) in the final dilution. Red cells were obtained from selected fowls which produced a constant inhibition titre with standard hyper-immune horse serum. Each fowl was used in rotation for approximately 3 weeks.

During the three months January–March, 1947, 202 pairs of sera were tested at the National Institute for Medical Research. The results are shown in Table III.

TABLE III.—*Results of Testing* 202 *pairs of Human Sera for Rise in Antibody.*

No. of pairs of serum tested.	Virus A (PR8). Rise in antibody.						Virus B (Lee). Rise in antibody.				
	0	×2	×4	×8	×16		0	×2	×4	×8	×16
202	81	57	39	12	13	202	161	40	—	1	—
Total	*Neg.*		*Pos.*			Total	*Neg.*		*Pos.*		
202	138		64			202	201		1		

Only one case of virus B infection was encountered, and that towards the end of the epidemic. These serological tests provided useful confirmation that the virus responsible for the outbreaks was of type A, at a time when the nature of the virus strain isolated in the chick embryo was by no means certain. Several patients from whom a virus was isolated showed only a two-fold rise in antibody, and a few no rise at all. This, in conjunction with the large number of sera with a two-fold rise in antibody (28 per cent) would seem to indicate that many people respond to an attack of influenza with less than a 4-fold rise in antibody.

At one hospital in North London an outbreak of influenza occurred in the second week of January and was investigated in some detail. Clinically the cases were typical of mild influenza. Attempts to isolate the virus in the chick embryo were at first unsuccessful. The serological response against PR8 was doubtful ; three showed a two-fold rise and two no rise in serum antibody. The throat washings which had been stored at —20° C. for a few weeks were then re-

examined. We inoculated 3 into ferrets and 5 into chick embryos, using both normal English eggs and eggs from White leghorn fowls brought to this country from Australia by the kindness of Professor F. M. Burnet, F.R.S. The results are shown in Table IV.

TABLE IV.—*Results of Inoculating Throat Washings from One Outbreak into Ferrets and Two Types of Chick Embryos.*

No. of cases.	Rise in human antibody.	Result of ferret inoculation.			Virus isolation in chick embryos.	
		Pyrexia.	Antibody response.			
			PR8	BAR ('47)	English	Australian
1	×2	103·5	×4	×16	+1/5	+3/5
2	×2	104	×2	×8	+1/5	+2/5
3	0	104·5	0	×4	+1/5	+3/5
4	×2	N.T.	N.T.	—	+1/5	+4/5
5	0	N.T.	N.T.	—	+2/5	+3/5

N.T. = Not tested.

These results show that out of 5 cases, 3 only showed a rise in antibody, and that only two-fold, and two produced a little pyrexia in a ferret. The antibody response in the ferrets was greater when tested with the current 1947 strain (BAR). The second attempt at virus isolation was more satisfactory; the Australian fertile eggs appeared to be more sensitive.

Besides testing human sera against standard PR8 antigen, we also carried out haemagglutinin tests against current (1947) viruses. The results were surprising. Many human sera showed an antibody rise against the standard PR8 antigen, but none or much less against the 1947 virus. Results against the latter were, furthermore, not readily reproducible. Professor Stuart-Harris informed us that he had encountered similar findings, which seemed to be associated with variations amongst the individual fowls furnishing red cells for test. Anderson, Burnet and Stone (1946) have lately described anomalous results associated with the use of different cell-batches. Such difficulties have not been met with in earlier A epidemics, and contrast with some American results (Salk, Menke and Francis, 1945); these workers found that haemagglutinin tests made against a recently isolated (Weiss) strain showed more significant rises than when PR8 antigen was used.

V. *Antigenic comparisons of strains.*

Representative strains from several of the outbreaks investigated were selected for special examination of their antigenic construction. Each of the following strains, BAR, JAC, WIL, EAS, EVA, ALL had had 3 amniotic and 2 allantoic passages before being used in this experiment. The method used for antigenic comparison was the modification of the red cell agglutination method described by Salk (1944), except that 2½ M.A.D. of antigen (as opposed to 4 M.A.D.) in the final dilution were used. Specific immune ferret sera were produced by the previous inoculation of ferrets with allantoic fluid of each strain. In each case a pre-inoculation sample of ferret serum was taken, so that the rise in antibody to each strain could be determined. In each of the strains tested

in this way there was a rise in PR8 antibody varying from 2–4 fold, but a rise in the homologous antibody of 8–16 fold. The individual strains all appeared to be closely related when tested by this method.

The original amniotic and allantoic fluids prepared from some of the early strains were not inhibited by specific PR8 serum to a titre higher than 64–128, and it seemed possible that these current strains might be atypical. Two of the current strains, JAC and Kunz, were compared with several classical A strains of influenza and the virus of swine influenza. The results of this antigenic comparison by the red cell method are shown in Table V.

TABLE V.—*To Show the Results of Antigenic Comparison between Several A Strains, Swine Virus and the Current Strain.*

Ferret antiserum.	Titres against virus.						
	PR8	JAC*	KUNZ*	W.S.	TAL	GAT	SW1
PR8	4096	64	64	N.T.	512	512	N.T.
*JAC	64	1024	1024	256	16	32	128
*NEW	64	1024	1024	128	32	64	128
W.S.	16	128	256	4096	32	512	256
TAL	2048	128	512	N.T.	4096	1024	N.T.
GAT	512	64	512	N.T.	16	8192	N.T.
SWI	32	256	256	256	256	256	4096

N.T. = Not tested. * = Current strain.

High titre agglutinin-inhibition was only produced by the interaction of a virus with its homologous antiserum; sera against standard strains all produced approximately the same degree of inhibition with the current strains JAC and Kunz. Convalescent human serum produced, as would be expected, less specific results than the ferret sera. Results of the haemagglutin tests were confirmed by a few neutralization tests in mice. The results of these experiments are therefore in harmony with those recorded in recent years from this and other laboratories; newly isolated strains are of varying degrees of antigenic remoteness from classical laboratory strains. The experiences of Glover and Andrewes (1943) and Hudson, Siegel and Markham (1943), in attempting to classify British strains of swine influenza, indicate the complexity of the problem.

DISCUSSION.

The behaviour of the influenza A virus during the winter 1946–47 closely resembled the behaviour of the B virus in the previous year. The virus first made its appearance in Britain in the first week in January, and for the next few weeks many localized epidemics were seen. Although the school population in this country was kept under closer observation than the remainder of the population, the higher attack-rate, which we noticed in the schools, does seem a quite definite fact. Professor F. M. Burnet reported similar findings from Australia with the A virus during the months April–June, 1946, and Dr. T. Francis in the U.S.A. had a similar experience during the first few months of 1947 (personal communications).

In the laboratory considerable success was achieved in the method of virus

isolation by the amniotic route. Previous lack of success with this method in this country can probably be attributed to several technical factors. In the first place the use of antibacterial substances, such as penicillin and sulphadiazine or sulphamerazine, has made filtration through a gradocol membrane unnecessary, and therefore the maximum amount of virus present in the washing can be inoculated into the embryo. Secondly, the period of incubation after inoculation was at least four days. The importance of this lies in the fact that there appears to be a rapid multiplication of virus at the first passage between the 3rd and 4th day after inoculation. In many cases it was found that eggs which were negative on the 2nd and 3rd day were positive on the 4th. Thirteen-day-old embryos were without any doubt the most sensitive for primary inoculation, and embryos of this age were used throughout the investigation. However, we encountered evidence suggesting that although this is an extremely simple and sensitive method for virus isolation, there is still a quantitative difference amongst eggs from various sources. There are many practical advantages in the amniotic method for virus-isolation and, from a technical point of view, there are not many difficulties. There remains always the possibility that our greater success in virus-isolation in 1947 was due mainly to a difference in the properties of the current virus as compared with those of former years. This view is supported by a recent report (Francis, Salk and Quilligan, 1947) that in an A outbreak in the U.S.A. in 1947 viruses could not be established in eggs, though they would infect ferrets, and though the authors had been successful with the egg-technique in previous outbreaks.

The serological tests showed that approximately 32 per cent of cases had a 4-fold rise or greater to PR8, and an equivalent number only a 2-fold rise. A similar proportion of rises against B virus (Lee strain) was encountered in the 1945–46 B outbreak. Several strains produced typical responses in ferrets, and 4 were adapted to mouse lung after 12 passages. In cross-immunity experiments with ferrets no antigenic differences amongst the 1947 strains could be detected under the conditions of the test. Attempts at comparing the antigenic structure of the strains by haemagglutination methods were not altogether successful. Several divergent results were obtained with various batches of red cells employed in the test, and it is certain that other techniques, including the mouse neutralization tests, should be employed before conclusions as to antigenic relationship are drawn.

SUMMARY.

From January to March, 1947, scattered outbreaks of a mild form of influenza A occurred in Britain ; the total incidence was low.

Serological tests of human sera against standard (PR8) antigen indicated that 30 per cent of cases were due to virus A. Tests against antigens made from homologous (1947) viruses gave anomalous results.

Much greater success was met with than in previous years in isolating viruses by amniotic inoculation of fertile eggs : 32/55 throat washings were positive. Properties of the 1947 viruses are described.

We wish to thank for their interest and help Dr. W. H. Bradley of the Ministry of Health, Dr. F. O. MacCallum and Professor C. H. Stuart-Harris ; also for technical assistance E. Owen and Miss E. Brodaty.

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THE ANTIGENIC EFFICIENCY OF *CL. WELCHII* EPSILON TOXIN AND TOXOID AFTER TREATMENT WITH TRYPSIN.

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THE action of trypsin on *Cl. welchii* Type D filtrates increases their toxicity, (Bosworth and Glover, 1934–1935), although the combining power or total antigen content, as measured by the Lf, is unaltered (Turner and Rodwell, 1943; Batty and Glenny, 1947). It was of both practical and theoretical interest to determine whether this alteration changed the immunizing efficiency of such toxic filtrates, and also to determine the effect of trypsin treatment on formol toxoid.

All antitoxic measurements recorded in this paper are given in terms of the unit which has always been used in these laboratories. We have been able to compare the Turner Rodwell unit with our own through the courtesy of these workers, and have found that 1 Australian unit = 1·33 Wellcome units.

The crude trypsin used in these experiments is an extract prepared by mincing 800 g. of fresh ox pancreas to which is added 2400 ml. tap water and 1000 ml. methylated spirit. The mixture is left at room temperature for 3 days, acidified by the addition of 0·1 per cent pure hydrochloric acid, stored at 4° C., and filtered before use.

Antigenic Activation of Toxoids by Trypsin.

Immunization of rabbits.

The filtrate from a broth culture of *Cl. welchii* Type D was divided into three portions; one was treated with 0·6 per cent formalin (0·24 per cent formaldehyde), the pH adjusted to 7·4 and incubated for 7 days, until it was non-toxic in doses of 0·5 ml. injected intravenously into mice, and in doses of 0·2 ml. given intracutaneously into guinea-pigs and rabbits. To the second portion, after similar treatment with formalin, was added 0·5 per cent of filtered crude pancreatic extract (trypsin) and the mixture was incubated at 37° C. for 45 minutes. These two additions were reversed in the third portion, trypsin being added before formalin.

Comparison of the antigenic efficiency of these three preparations was made by measuring the antitoxic content of the serum taken from rabbits 10 days after the second of two subcutaneous injections of 1·0 ml. given at an interval of 4 weeks. The results given in Table I show that trypsinized toxoid is more efficient than untreated toxoid, the geometric means of the antitoxic values of groups of 5 rabbits being 0·912 and 0·126 unit per ml. respectively. Treatment of toxoid with trypsin (second portion) did not alter the combining power, but the third portion toxoided after trypsin treatment suffered loss in total antigenic content as shown by the increase in Lf from 0·08 ml. to 0·12 ml.; in spite of this loss all 5 rabbits immunized with this material produced more antitoxin than any of the 10 immunized with the other portions. It is possible, however, that the increased antigenic efficiency may have been due to slight residual toxicity outweighing the loss in combining power. A similar experiment with a 5-day growth did not give such conclusive results, but it would appear that trypsin treatment improved antigenic efficiency to a slight degree in spite of considerable loss in combining power.

TABLE I.—*Showing the Immunity Response of Rabbits to Two Doses at an interval of 4 weeks of* 1·0 *ml. of* Cl. welchii ε *Toxoid or Anaculture, Before and After Treatment with Trypsin.*

Antigen.	Lf in ml.	Number of rabbits with given antitoxic titres 10 days after the second injection. Units per ml.										Geometric(*d*) Mean.
		0·005(*c*)	0·01.	0·02.	0·05.	0·1.	0·2.	0·5.	1·0.	2·0.	5·0.	
Toxoid												
24 F	0·08	1	–	–	–	1	3	–	–	–	–	0·126
24 F T	0·08	–	–	–	–	–	1	1	3	–	–	0·912
24 F T (*a*)	0·12	–	–	–	–	–	–	–	–	3	2	4·35
120 F	0·08	2	–	1	1	–	–	1	–	–	–	0·038
120 F T	0·18	1	1	–	–	1	1	1	–	–	–	0·079
120 T F	0·18	1	–	2	–	–	1	1	–	–	–	0·089
120 T F T	0·20	–	1	1	1	2	–	–	–	–	–	0·058
Anaculture												
Untreated	0·025	1	1	1	2	1	3	1	–	–	–	0·093
Treated	0·026	–	–	–	1(*b*)	–	2	3	–	4	–	0·871

Note.—In this and other tables antigens are described by a number indicating the length of growth in hours and the order of modification with formaldehyde F or treatment with trypsin T.

(*a*) This batch was slightly toxic, and some loss of antigen occurred (as judged by Lf) when formalin was added after trypsin treatment.

(*b*) This rabbit gave birth to a litter of young between the two injections.

(*c*) Values under 0·01 are counted as 0·005 when estimating the geometric mean.

(*d*) Any rabbit with a given recorded value contained at least this amount but less than the next higher amount; in calculating the geometric mean such titre is taken as the mean between the recorded titre and the next higher one.

The routine ovitoxicus antigen, used for the immunization of sheep, has been an anaculture prepared from a whole culture of *Cl. welchii* Type D grown in a peptone meat infusion broth, with added meat, for 4 days at 37° C. The meat is removed, 0·6 per cent formalin is then added, the pH adjusted to 7·4, and the anaculture returned to the incubator and left until non-toxic. The immunizing value of such a preparation before and after treatment with trypsin was tested on rabbits in the same way as the toxoid recorded above. Table I shows rabbits immunized with trypsin-treated anaculture produced nearly 10 times as much antitoxin as rabbits immunized with untreated anaculture.

TABLE II.—*Showing the Immunity Response of Guinea-pigs to Two Doses at an interval of* 4 *weeks of* 1·0 *ml. of* C. welchii ε *Toxoid, Before and After Treatment with Trypsin.*

Toxoid.	Lf. in ml.	Number of guinea-pigs with given antitoxic titre 10 days after the second injection. Units per ml.									Geometric mean.
		0·005.	0·01.	0·02.	0·05.	0·1.	0·2.	0·5.	1·0.	2·0.	
6 F	0·113	3	–	3	3	–	1	–	–	–	0·0346
6 F T	0·113	1	–	–	–	2	2	1	2	–	0·258
6 T F	0·158	2	3	1	–	–	–	–	3	1	0·091
24 F	0·08	–	1	1	3	2	1	–	–	1	0·114
24 F T	0·08	–	1	–	–	–	4	1	–	3	0·527
(*a*)24 T F	0·12	5	–	–	–	–	–	2		2	0·0834

(*a*) Slightly toxic.

Immunization of guinea-pigs.

Table II records experiments on guinea-pigs treated in a similar manner to the rabbits, and confirms that treatment with trypsin increased the immunizing efficiency of filtrates from 24 hours' growth that had been rendered non-toxic by formalin. The geometric means of the antitoxic values of guinea pigs immunized with treated and untreated toxoids were 0·527 and 0·114 unit per ml. respectively. The third portion, treated with trypsin before toxoiding, gave very irregular results, probably due to the slight toxicity being sufficient to lower the condition of some of the guinea-pigs so that they failed to respond adequately, while other guniea-pigs, less affected, responded well. Three similar portions were prepared from a 6-hour culture; again the toxoid treated with trypsin was definitely a better antigen than untreated toxoid. The geometric means of the antitoxic values were 0·258 and 0·0346 unit per ml. respectively: the third portion gave irregular results. A similar experiment with a 5-day growth gave inconclusive results both in rabbits and in guinea-pigs; the combining power of the trypsinized portions was reduced to less than one-half that of the untreated toxoid.

Immunization of horses.

It has been for many years the custom at these laboratories to choose for hyperimmunization against any toxin those horses whose serum has been found to contain the appropriate antitoxin. For the preparation of antitoxins which do not occur naturally in horses, hyperimmunization is delayed until basal immunity has been established, that is, until some months after a preliminary treatment consisting either of a short course of up to five injections of an appropriate antigen, or of two doses given at an interval of 4 weeks. For many years

the antigen chosen for the two-dose method has usually been alum precipitated toxoid (A.P.T.). Advantage was taken of this routine procedure to compare alum precipitates from untreated and from trypsin-treated epsilon toxoid. Alternate horses were injected with the two types of antigen, and the sera obtained by bleeding the horses 10 days after the second injection were tested for antitoxic content. The filtrate from a 24-hour culture of *Cl. welchii* Type D was rendered non-toxic by incubating for 7 days after the addition of 0·6 per cent formalin. One half was precipitated by the addition of 2 per cent potash alum and was left at room temperature overnight; the supernatant liquid was drawn off and the precipitate from 50 litres resuspended in 14 litres of saline. To the other half was added 5 per cent crude trypsin, and it was then incubated at 37° C. for 1 hour before precipitation in a like manner. It was found that 7 out of 12 horses given the A.P.T. from trypsin-treated toxoid reached 10 units or more compared with 2 out of 13 given A.P.T. from the untreated material. The corresponding geometric means were 11·31 and 4·58 units per ml.

Immunization of lambs.

Similar results, showing the increased antigenic efficiency of trypsinized toxoid, have been obtained by Batty and Thomson (in course of publication) in an experiment devised to determine the most suitable antigen, and the best course of injections for field use in the protection of lambs against pulpy kidney. As expected, the best results were from the use of an alum precipitate of trypsin-treated anaculture.

Antigenic Activation of Toxin by Trypsin.

Hyperimmunization of horses.

As already mentioned, horses receive some preliminary treatment to establish basal immunity before the commencement of the first course of hyperimmunization. During the first course horses are given two intramuscular injections each week consisting of increasing doses of toxin. At the end of 4 or 5 weeks experimental bleedings are taken and tested for epsilon antitoxic content by the flocculation method; further samples are taken frequently, usually weekly, and occasionally the results of flocculation tests are checked by *in vivo* tests. When it appears that the antitoxic content has almost stopped increasing the horse is bled. Subsequent immunizations follow a completely different course; 5 injections are given on alternate days consisting usually of one of 50 ml. followed by one of 100 ml. and then 3 of 150 ml. of toxin, to each litre of which has been added 40 ml. of 10 per cent potash alum.

The flocculation test has a distinct value in *Cl. welchii* ε immunization apart from the saving of animals by preliminary titrations. Barr and Glenny (1945) have shown that there is some relation between the quantity and quality of antitoxin produced in horses; quality is best measured by the "serum ratio," i.e. the apparent *in vivo* value of an antitoxin divided by the *in vitro* value. Table III shows that there is a tendency for the serum ratio of *Cl. welchii* ε antitoxin to increase slightly with increased antitoxic value. Of greater practical importance is our finding that horses with a low serum ratio at the end of (and probably during) the first course of immunization do not subsequently produce high value antitoxin. The figures given in Table IV show that no horse out of 11

with a serum ratio of less than 1·0 produced over 500 units per ml. at the end of the first course of immunization, while the majority of horses with a ratio above 1·0 subsequently produced over 500 units per ml. and 10 out of 32 over 800 units per ml.

TABLE III.—*Showing the Serum Ratios of* Cl. welchii ε *Sera divided according to the Antitoxic Values and the Stage of Immunization reached.*

Course of immunization.	Antitoxic value in units per ml.					Average ratio.	Number of bleedings.
	<200	200–300.	300–500.	500–800.	Over 800.		
1st	1·05	1·05	1·13	1·15	1·37	1·10	69
2nd	1·14	1·14	1·11	1·13	1·23	1·14	55
3rd	1·12	1·09	1·14	1·30	1·17	1·14	35
4th or later	1·18	1·08	1·15	1·21	1·28	1·17	31
All courses	1·09	1·09	1·13	1·16	1·22	1·13	..
Number of bleedings	40	40	51	39	20	..	190

TABLE IV.—*Showing the Relation between the Serum Ratio of* Cl. welchii ε *Antitoxin at the End of the First Course of Immunization and the Antitoxic Value subsequently reached by the same Horse.*

Serum ratio after 1st immunization.	Number of horses. units per ml.					Number of horses. > 500 units per ml.		
	Under 200.	200–300.	300–500.	500–800.	Over 800.	Total.	Number.	Per cent.
Under 1·0	5	3	3	–	–	11	0	0
1·0 to 1·099	1	3	3	7	5	19	12	63
1·10 or greater	–	–	2	6	5	13	11	84

It must be pointed out that the serum ratios are comparative and not absolute, because the actual value of the ratio depends upon the standard serum chosen for *in vitro* testing. General results suggest that our chosen *in vitro* unit should have been fixed at least 10 per cent greater than it was, so that the average serum ratio of all well immunized horses would be 1·0 or slightly greater. Because considerable increase in antitoxic titre followed the use of activated toxin for the hyperimmunization of horses, a return to the use of unactivated toxin was not economical; consequently comparisons are available between the values reached after immunization with unactivated followed by activated toxins but not the reverse. In Tables V and VI comparison is made between the

TABLE V.—*Showing the Number of Horses Increasing or Decreasing in* Cl. welchii ε *Antitoxic Value from the 1st course of Immunization to the 2nd, divided according to the Type of Toxin used.*

	Toxin used.		
1st immunization :— 2nd ,, Alteration in antitoxic values (percentage).	Unactivated. Unactivated.	Activated. Activated.	Unactivated. Activated.
	Number of horses.		
Decrease 35–45	1	–	–
20–35	5	1	–
0–20	9	6	–
Increase 0–20	10	2	–
20–50	12	7	2
50–80	3	3	2
80–120	4	2	3
120–170	0	1	2
170–230	1	–	1
Over 230	–	–	1

values after successive immunizations when the toxin is changed from unactivated to activated, and the values when the type of toxin remains unchanged. Table V shows that most horses produce more antitoxin after the second than after the first immunization, especially if the toxin is changed from unactivated to activated. Table VI shows that there is a tendency for values of bleedings after subsequent immunizations to decrease (31 out of 47) when unactivated toxin is used continually; values are maintained when activated toxin is used (39 decrease, 42 increase), but most horses increase in value when the toxin is changed from unactivated to activated (8 out of 13 increase more than 50 per cent); those that decreased in value were low value horses.

TABLE VI.—*Showing the Number of Horses Increasing or Decreasing in* Cl. welchii ε *Antitoxic Value from One Course of Immunization (other than the first) to the Next, Divided according to the Type of Toxin used.*

	Toxin used.		
$(n+1)$ immunization :— $(n+2)$ "	Unactivated. Unactivated.	Activated. Activated.	Unactivated. Activated.
Alterations in antitoxic values (percentage).	Number of horses.		
Decrease over 45	3	4	–
35–45	4	11	–
20–35	10	12	1
0–20	13	12	2
Increase 0–20	11	15	0
20–50	3	17	2
50–80	0	7	2
80–120	2	1	4
120–170	–	2	1
170–250	–	–	1

Although as a general rule more antitoxin is produced when horses are immunized with stronger toxin, the improved antigenic efficiency of trypsin-treated toxin is such that relatively weak toxin so activated is a better antigen than much stronger unactivated toxin. Table VII records the antitoxic values of 12 horses after immunization with one or other of two similar batches of unactivated toxin of approximately the same strength, and the values reached

TABLE VII.—*Showing the Changes in* Cl. welchii ε *Antitoxic Titres of Horses after Successive Immunizations with Unactivated followed by Activated Toxin.*

Serial number of immunization.		Toxin used :— (Lf).	Unactivated. 0·06 to 0·09 ml.	Activated. 0·12 to 0·15.	Percentage increase or decrease in value.
With unactivated toxin.	With activated toxin.		Antitoxic values of individual horses in units per ml.		
2	3		260	225	−14
3	4		280	230	−18
3	4		575	425	−26
5	6		195	300	+54
2	3		475	750	+58
4	5		380	610	+60
4	5		430	800	+86
2	3		140	270	+93
4	5		165	330	+100
4	5		52	105	+102
2	3		925	2100	+127
5	6		575	1550	+169
Geometric mean			293	452	+54

after the next immunization with a much weaker activated toxin. Epsilon toxin is somewhat unstable ; the table records the results of the Lf determinations on the toxins immediately before use, and the lowest value (highest Lf) of those recorded from tests that are always made by us on samples taken after the last injection has been made from each bottle.

It has already been shown in Table VI that horses do not, on the average, tend to improve in antitoxin production with successive immunizations after the second ; Table VII shows that out of 12 horses given weak activated toxin, 3 gave titres between 14 and 26 per cent lower and 9 gave titres between 58 and 169 per cent higher than the values after the previous immunization with stronger unactivated toxin. As a control to these results we record that 11 horses were immunized with strong unactivated toxin of the same strength as that recorded above and yielded an average (geometric mean) value of 394 units per ml., while 15 horses immunized at the same time with a weaker unactivated toxin, similar in strength to the activated given above, yielded an average of 228 units per ml.

Table VIII summarizes the results of 164 immunizations with activated toxin and 167 with unactivated toxin carried out at the same time or shortly before those with activated toxin. The average combining power of the various batches of each type of toxin was about the same. The advantage of using activated toxin is not so great during the first immunization, but for the 3 groups, 2nd, 3rd, and subsequent immunizations, the gain is very nearly 100 per cent; the geometric mean of values following unactivated toxin were all between 272 and 280 units, compared with between 510 and 535 for activated toxin.

TABLE VIII.—*Comparing the* Cl. welchii ε *Antitoxic Values of Horses Hyperimmunized with Unactivated and Activated Toxin.*

	Geometric mean of antitoxic values in units per ml.		Number of horses.	
Toxin used :— Course of immunization.	Unactivated.	Activated.	Unactivated.	Activated.
1st	268	376	63	27
2nd	274	533	51	39
3rd	280	510	25	27
4th or later	272	535	28	71
All	274	506	167	164

Results from a single horse (4563) show the effect on the antitoxic response of using strong toxin and activated toxin for hyperimmunization. The first immunization yielded 525 units followed by 1150 and 1050. Subsequent immunization, still with average strength unactivated toxin, yielded less antitoxin until the 8th immunization gave 750 units per ml. Strong toxin produced by partial concentration was used for the 9th immunization and yielded 900 units, for the 10th course activated toxin of the same strength was used and resulted in a production of 1150 units per ml. The partially concentrated toxin was used again and gave 675 units ; finally a return to activated toxin yielded 1200 units per ml.

The distribution of values after immunization with unactivated or activated toxin given in Table IX shows that horses fall into two main groups—good and bad immunizers. Values reached after the first immunization show that for the 50 per cent bad or slow immunizers the use of activated toxin is no better, probably worse, than the use of unactivated toxin, but for the good immunizers

definitely better results are obtained with activated toxin. For subsequent immunizations the ascendancy of activated toxin is unquestioned for all types of horses.

TABLE IX.—*Showing the Distribution of* Cl. welchii ε *Antitoxic Values of Horses Immunized with Unactivated and Activated Toxin, divided according to whether the Immunization was the First Long Course or a subsequent Rapid Course of Toxin with Alum.*

Antitoxic values in units per ml.	Percentage number of bleedings of different antitoxic values.			
	After 1st course of immunization with—		After subsequent courses of immunization with—	
	Unactivated.	Activated.	Unactivated.	Activated toxin.
Under 150	9·5	22·3	18·3	1·5
150–200	11·1	7·4	7·7	5·1
200–240	9·5	14·8	11·5	2·9
240–300	19·1	7·4	12·5	5·1
300–360	20·7	3·7	11·5	6·7
360–450	11·1	7·4	12·5	11·7
450–550	8·0	7·4	10·5	16·1
550–650	3·0	11·1	4·8	13·1
650–800	8·0	18·5	6·7	13·8
800–1000	..	..	3·0	10·2
1000–1200	..	..	1·0	4·3
1200–1500	..	..	..	5·8
Over 1500	..	..	..	3·7
Under 300	49·2	51·9	50·0	14·6
300–450	31·8	11·1	24·0	18·4
Over 450	19·0	37·0	26·0	67·0
Total number of immunizations	63	27	104	137

Materials used in these experiments were mostly routine preparations or special modifications made for us by Mr. J. MacSween, to whom we offer our grateful thanks.

SUMMARY.

1. Treatment with trypsin increases the antigenic efficiency of *Cl. welchii* ε toxoid.

2. The alum precipitate from trypsin-treated epsilon toxoid is a better antigen than the precipitate from untreated toxoid.

3. The use of trypsin-treated epsilon toxin for the hyperimmunization of horses almost doubles the yield of antitoxin.

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THE EFFECTS OF SHORT-TERM CHANGES IN DIETARY PROTEIN ON THE RESPONSE OF THE LIVER TO CARBON TETRACHLORIDE INJURY.

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THE nutritional condition of an animal greatly determines its resistance to toxic halogenated hydrocarbons. The livers of dogs which have been fasted or given an all-fat diet for as short a period as four days are highly susceptible to chloroform or carbon tetrachloride injury. On the other hand, diets rich in protein or, still more strikingly, diets rich in carbohydrate are highly protective when given for similar periods (Davis and Whipple, 1919 ; Davis, 1924). Corresponding effects of diets rich in fat or carbohydrate have been observed in chloroform poisoning of rats (Opie and Alford, 1915). Although a diet high in carbohydrate and low in protein is highly protective when given for a short period only, protein depletion renders the livers of dogs very sensitive to the action of chloroform (Miller and Whipple, 1940, 1942). Rats fed on diets low in casein for several weeks are more susceptible to chloroform and carbon tetrachloride than animals on a diet adequate in protein (Goldschmidt, Vars and Ravdin, 1939 ; György, Seifter, Tomarelli and Goldblatt, 1946).

In view of the fact that the protein content of the diet determines the amount of labile cytoplasm (protein + phospholipin + nucleic acid, PPN) present in the liver cell (Kosterlitz, 1944, 1947), it was of interest to examine whether the severity of the liver injury caused by a single administration of carbon tetrachloride could be correlated with the presence or absence of labile PPN. It was intended also to test whether or not carbon tetrachloride caused a breakdown of labile liver PPN. In order to vary the amount of labile PPN, rats were placed on a protein-free, an 18 per cent or a 54 per cent casein diet for three days prior to a single injection of carbon tetrachloride. This short preliminary period produced the desired changes in PPN without causing a significant protein depletion of the body as a whole in the protein-free fed rats. The diets contained sufficient choline to prevent fatty infiltration of the livers of the control rats, which would increase the sensitivity to halogenated hydrocarbon (Goldschmidt *et al.*, 1939).

METHODS.

Analytical methods.

These have already been described in a previous paper (Kosterlitz, 1947).

Experimental procedure.

Male hooded rats, three months old, of the Rowett Institute strain, were used. Their average body weight was 293 g., ranging from 251 to 344 g. For

each experimental treatment groups of four rats were selected with a mean body weight varying between 280 and 315 g. The basal diet (Kosterlitz, 1947) consisted of 2 per cent agar, 3 per cent salts, 25 per cent sucrose, 60 per cent potato starch and 10 per cent lard. The lard was supplemented with vitamins A, D, and E ; the B vitamins were given partly in the form of the pure chemical compounds and partly as liver concentrate. The casein used was fat- and vitamin-free (Glaxo Laboratories Ltd.), and had 14·2 per cent N. The carbon tetrachloride (B.D.H. Analar) was given as a single subcutaneous injection of 0·2 ml./100 g. initial body weight on the third day of the experimental period. The different groups of animals were killed 4½ to 5, 24, 48 and 72 hours after the administration of carbon tetrachloride.

Histological technique.

Liver tissue was fixed in 5 per cent neutral formaldehyde in 0·9 per cent NaCl. Paraffin sections were stained with haemalum and eosin, and frozen sections with scarlet R and haemalum, or sudan black.

RESULTS.

Effect of carbon tetrachloride on food intake.

In the preliminary feeding period before the injection of carbon tetrachloride, all rats, with the exception of a small number on the 54 per cent casein diet, consumed at least 6·5 g. food/100 g. body weight. The animals which voluntarily restricted their food intake below 6·5 g. are not included in the general results, but will be dealt with separately. After injection of carbon tetrachloride, the rats on the 18 per cent casein diet scarcely reduced their food consumption, those on the 54 per cent casein diet reduced it by 16 per cent, and those on the protein-free diet by 33 per cent. The injected rats fed on the 18 per cent and 54 per cent casein diet gained weight more slowly than controls on the same diets, while those on the protein-free diet lost weight more rapidly than the corresponding controls.

Chemical changes in the liver.

Weight and protein + phospholipin + nucleic acid (PPN) contents of livers.— The control animals showed the following changes (Fig. 1) : On the 18 per cent casein diet both liver weight and PPN remained constant ; on the 54 per cent casein both values rose during the first three days of the diet and then remained constant, while on the protein-free diet PPN showed a decrease, continuing during the whole experimental period of seven days (Campbell and Kosterlitz, 1946). The relative constancy of the liver weight during the latter half of the protein-free period was due to the increased deposition of glycogen (Fig. 2).

Subcutaneous injection of carbon tetrachloride caused in each dietary group a rise of both liver weight and PPN. In the rats on both the 18 and 54 per cent casein diets the values reached their maximum 48 hours after carbon tetrachloride, and at 72 hours were still above normal. In the rats on the protein-free diet, the rise in PPN after carbon tetrachloride injection was rather more pronounced than in the two other groups, and the difference in PPN between the control and the injected rats remained approximately constant during the three days of the experiment. It is further of importance to note that, within each

dietary group, the protein, phospholipin and nucleic acid indices altered only very little after injection of carbon tetrachloride. As was to be expected (Kosterlitz, 1947), the protein index (protein × 100/PPN) was highest in the rats fed on the 54 per cent casein diet (80·6), intermediate in those fed on the 18 per cent casein diet (79·7), and lowest in those fed on the protein-free diet (78·6), while the nucleic acid index (nucleic acid × 100/PPN) was lower in the 54 per cent (5·45) and 18 per cent casein (5·55) than in the protein-free group (6·5). After injection of carbon tetrachloride the indices remained almost constant; there was a tendency for the protein indices to fall and the nucleic acid indices to rise very slightly. The former were 79·5, 79·1 and 78·1, and the latter 5·8, 5·95 and 6·9 for the 54 per cent casein, 18 per cent casein and protein-free groups respectively. The phospholipin indices showed no consistent variations (14·8).

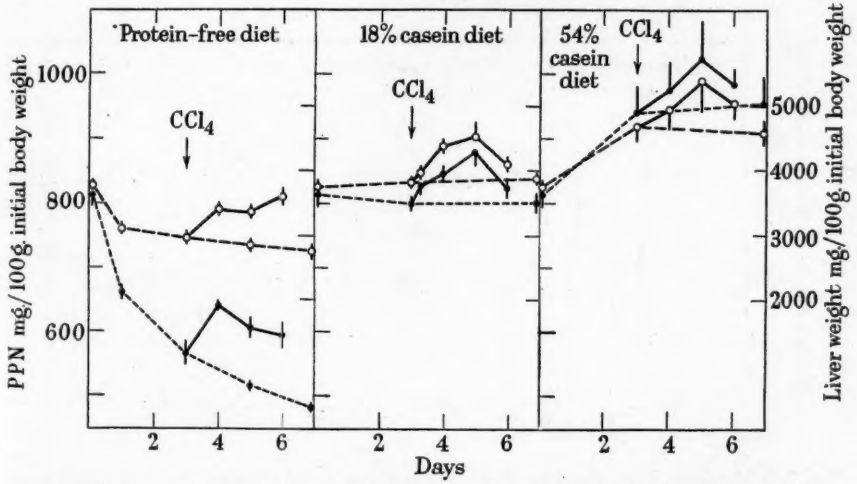


FIG. 1.—Changes in liver weight and PPN in rats on different diets with and without injection of carbon tetrachloride. Each value represents the mean obtained from 4 rats. The vertical lines through the points and circles represent twice the S.E.M., and in some instances, in order to avoid confusion, were drawn in one direction only.

Liver weight
○— — — — —○ control rats.
○————————○ experimental rats.
PPN
● - - - - - - - ● experimenta rats.
●————————● control rats.

Glycogen content of the liver.—Liver glycogen fell within 5 hours after the injection of carbon tetrachloride to levels which appeared to be almost independent of the diet (Fig. 2). There were variations in the course of the experiments which were, however, insignificant with the exception of the three-day value in the rats on the 18 per cent casein diet.

Neutral lipid content of the liver.—The neutral lipid content of the livers of the control animals varied only little; it was lowest in the protein-free group and highest in the 54 per cent casein group (Fig. 3). No significant change was observed during the first 5 hours after carbon tetrachloride. From the

24-hour value onwards a steep rise was found on all diets. All values found in the rats on the 54 per cent casein diet were significantly greater than those obtained from the animals on the protein-free diet. The 24-hour value of the 18 per cent casein group was higher than the corresponding value of the protein-free group, while no significant differences between these two diets were found at 48 and 72 hours. Both the 24- and 48-hour values of the 54 per cent casein group were higher than those of the 18 per cent casein group, while the 72-hour values did not differ significantly. It would appear then that the deposition of neutral lipid was favoured by a high casein intake.

Water content of the liver.—Fenn (1939) adduced evidence that in rats 2·33 ml. of water are deposited for each 1 g. of glycogen, of which 0·8 ml. is extracellular. Fenn and Haege (1940) found, by means of partial regression equations,

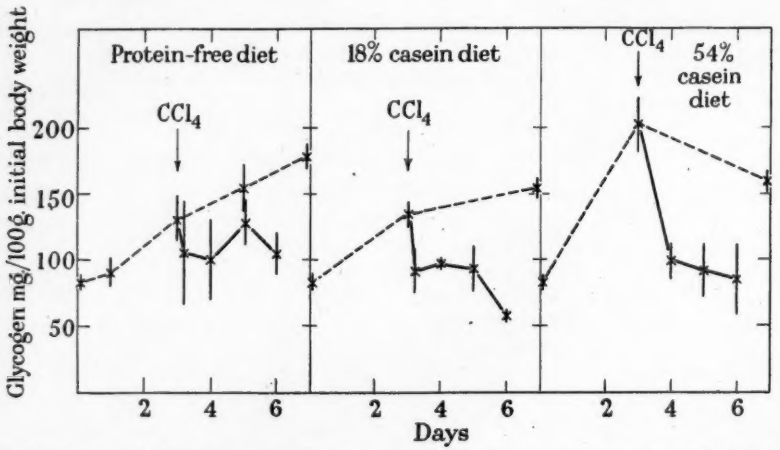


FIG. 2.—Changes in liver glycogen of rats on different diets with and without injection of carbon tetrachloride. Each point represents the mean obtained from 4 rats. The vertical lines represent twice the S.E.M.

× — — — — — × control rats.
× ———————— × experimental rats.

that in cats 1·57 ml. was deposited with 1 g. glycogen and 3·29 ml. with 1 g. protein. No water was deposited with lipid. The values were calculated on the assumption that all the water present in the liver was accounted for by that deposited with protein and glycogen.

The results of a calculation of the partial regression coefficients from the data of present and previous experiments (Kosterlitz, 1947 ; Campbell and Kosterlitz, 1948) are summarized in Table I. The values obtained for the deposition of water with glycogen did not differ for any of the groups of animals. There was no significant variation in the water calculated to be associated with PPN except in the female high-protein groups, for which the regression coefficient was significantly increased. The constant "a," which probably accounts for extracellular water, appeared to be higher on the low protein groups than in the normal and fasted groups.

TABLE I.—*The Partial Regression Coefficients for Water* (y) *on PPN* (x_1) *and Glycogen* (x_2). $Y = a + b_1x_1 + b_2x_2$. *The constant* a *denotes water which is not deposited with either PPN or glycogen.*

Diet (number of animals).	a.	b_1.	b_2.
(1) High-protein (20 ♀)	−243	3·25 ± 0·27 (S.E.M.)	1·86 ± 0·61
(2) Normal (45 ♀)	326	2·69 ± 0 21	1·55 ± 0·28
(3) Low and protein-free (56 ♀)	594	2·36 ± 0·18	1·88 ± 0·16
(4) Fasted (23 ♀)	190	2·82 ± 0·34	—
(5) (2)–(4) pooled	—	2·55 ± 0·13	1·80 ± 0·14
(6) Normal and protein-free (25 ♂)	403·5	2·41 ± 0·10	2·53 ± 0·33

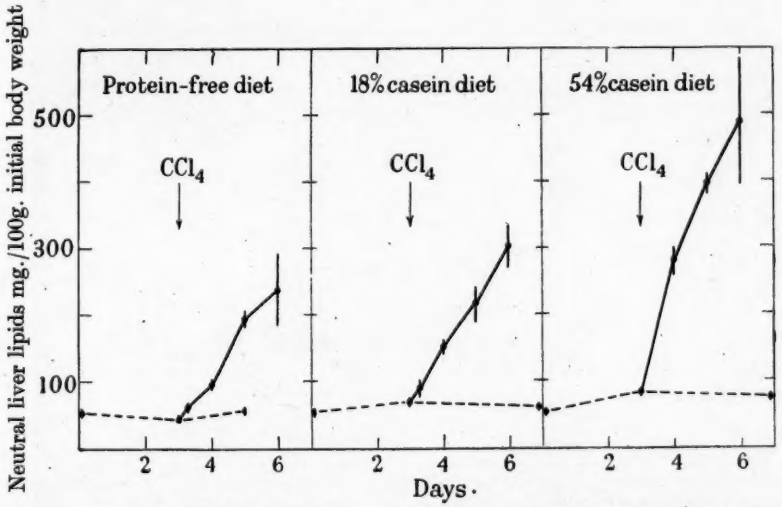


FIG. 3.—Changes in neutral liver lipids of rats on different diets with and without injection of carbon tetrachloride. Each value represents the mean obtained from 4 rats. The vertical lines represent twice the S.E.M.

●— — — — —● control rats.
●————————● experimental rats.

In order to obtain some measure of the amount of water associated with PPN, the ratio of non-glycogen water/PPN was determined for rats on the different diets, with and without carbon tetrachloride. For these calculations, the factor suggested by Fenn (1939), 2·33 ml. H_2O/1 g. glycogen was used, which did not differ significantly from the partial regression coefficient for glycogen found for our male rats (2·53 ± 0·33). The constant "a" was neglected so that part of the non-glycogen water must be assumed to be extracellular. It was found that the ratio non-glycogen water/PPN was the same for the controls on the stock, 18 per cent and 54 per cent casein diets (2·93 ± 0·014 ; 20 cases), while it was raised on the protein-free diet (3·17 ± 0·018 ; 8 cases). After administration of carbon tetrachloride a very marked rise in the ratios above the control values

was observed in each group (Fig. 4). Within each dietary group the values did not vary significantly with the exception of a rise on the third day in the protein-free group. The pooled means were as follows; 3·50 ± 0·061 (16 cases) for the protein-free group, 3·25 ± 0·060 (16 cases) for the 18 per cent casein group and 3·22 ± 0·064 (12 cases) for the 54 per cent casein group, the first value being significantly higher than the second and third.

Histological changes in the liver.

The histological changes occurring in the liver of the rat after a single oral or subcutaneous administration of carbon tetrachloride have been described in detail by Lacquet (1932), and Cameron and Karunaratne (1936). The most outstanding features are central necrosis with fatty degeneration and the appearance of large balloon-like cells; the contents of these consisted of small dark nuclei and a few threads between the nuclei and the periphery. Lacquet believed

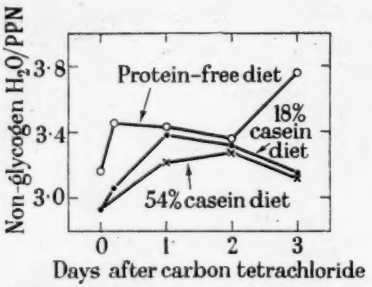


FIG. 4.—Non-glycogen water/PPN ratios in rats fed on different diets before and after injection of carbon tetrachloride.

that they contain fat, while Cameron and Karunaratne found neither fat, lipoid nor glycogen, and considered that these cells represent hydropic degeneration. Increased frequency of mitosis was observed from the first to the third day.

In the experiments described in the present paper the histological appearances varied considerably with the diet fed to the rats. The animals fed on the 18 per cent casein diet showed the typical changes described above (Fig. 5–7).

EXPLANATION OF PLATE.

Paraffin sections of livers fixed in neutral 5 per cent formaldehyde solution in 0·9 per cent NaCl and stained with haemalum-eosin. × 70.

FIG. 5.—Liver from a rat fed on the 18 per cent casein diet for 1 week.

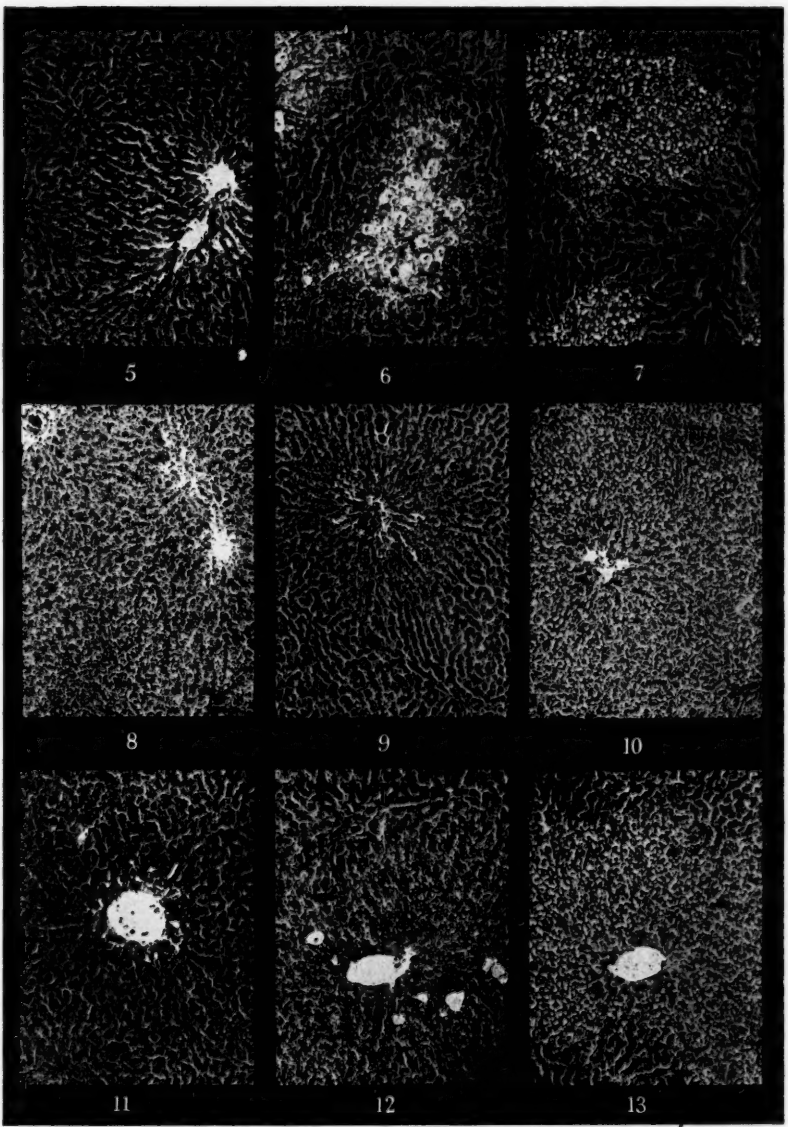
FIG. 6, 7.—Livers from rats fed on the 18 per cent casein diet; 24 and 48 hours after carbon tetrachloride, injected on the 3rd day of the experimental period.

FIG. 8.—Liver from a rat fed on the protein-free diet for 5 days.

FIG. 9, 10.—Livers from rats fed on the protein-free diet; 24 and 48 hours after carbon tetrachloride, injected on the 3rd day of the experimental period.

FIG. 11.—Liver from a rat fed on the 54 per cent casein diet for 3 days.

FIG. 12, 13.—Livers from rats fed on the 54 per cent casein diet; 24 and 48 hours after carbon tetrachloride, injected on the 3rd day of the experimental period.



Campbell and Kosterlitz.

After 24 hours a considerable number of large hydropic cells were found in the centre of the lobule with congestion of the central vein. In the midzone the cells had more or less pyknotic nuclei and fat globules in their cytoplasm. There was increased frequency of mitosis in the periportal space. After 48 hours the hydropic cells had disappeared in 3 out of 4 animals. In the central and middle zones of the lobules there were now, apart from some hyaline material, irregularly arranged cells containing much fat and showing little or no cytoplasmic basophilia. After a further 24 hours the histological appearance was similar; there was a further increase in the fat content. Mitoses were less numerous on the second and third days. Some infiltration of round cells was present on the third day.

In rats which had received the protein-free diet for 3 days before the injection of carbon tetrachloride the changes were very much less pronounced (Fig. 8–10). Hydropic cells were found in only one out of 12 animals, the arrangement of the cell strands was less disturbed, particularly on the first and second day, and there was less fat in the cells. With the exception of one rat, there was no frank necrosis. Mitoses were scanty with the exception of the one rat showing necrosis. There were round cells in the central part of the lobule on the third day.

The changes observed in the rats fed on the 54 per cent casein diet were somewhat similar to those found in the 18 per cent casein group. There were, however, a smaller number of hydropic cells, and a much larger accumulation of fat in the cells of the central and middle zones. Necrosis in the central parts occurred later, and then was not so pronounced as in the 18 per cent casein group. Some round cells were found in the central zone on the third day.

In all groups of animals there was an early decrease in basophil granules in the cells of the central parts of the lobules—a fact which has already been pointed out by Cameron and Karunaratne (1936), and by Rosin and Doljanski (1946). The diffuse basophilia in the periportal area appeared to be increased in intensity after carbon tetrachloride. Thus, in sections stained with haemalum only, there was a very marked contrast between the affected central area of the lobule without cytoplasmic staining and the peripheral area with the cytoplasm stained intensely blue.

TABLE II.—*Composition of the Livers of Rats on the 54 per cent Casein Diet who voluntarily restricted their Caloric Intake before CCl_4 Administration.*

Diet (number of animals).	Changes in weight before / after CCl_4. g.	Mean daily food intake before / after CCl_4. g.	Liver weight. g.	Glycogen. mg. /100 g. initial body weight.	Neutral lipids. mg. /100 g. initial body weight.	PPN. mg. /100 g. initial body weight.	Non-glycogen water/PPN.
54% casein 3 days							
+CCl_4 1 day	+1	5·8					
(1)	−2	3·0	5·17	36	168	703	5·35
CCl_4 2 days	+2	5·8					
(1)	−19	0·6	4·65	23	221	638	5·27
CCl_4 3 days	−4	5·6	4·26	65	292	731	3·91
(3)	−21	2·3	±0·26 (S.E.M.)	±40	±52	±31	±0·059

A small number of rats fed on the 54 per cent casein diet voluntarily restricted their calorie intake during the preliminary feeding period ; after the injection of carbon tetrachloride their food intake became very small indeed (Table II). These animals suffered considerable losses in weight ; owing to the small calorie intake the PPN and neutral lipid contents of their livers was considerably smaller than those of the rats eating adequate amounts of the 54 per cent casein diet. The non-glycogen water/PPN ratios were strikingly raised. Histologically, the hydropic and necrotic changes exceeded in severity those found in the 18 per cent casein group.

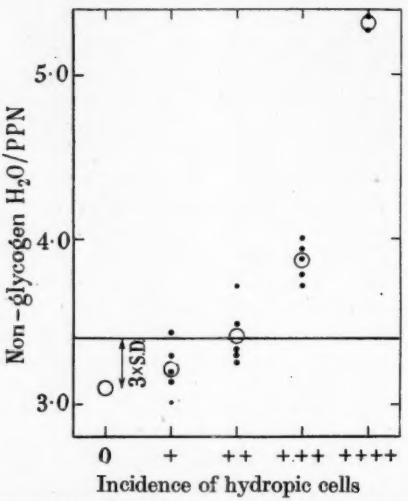


FIG. 14.—Correlation between the non-glycogen/PPN ratios and the incidence of hydropic degeneration of liver cells. The large circles represent means (3 × S.D. = 3 times the standard deviation of the non-glycogen/PPN ratio of livers without hydropic cells).

Correlation between histological appearance and chemical composition.

As was to be expected, a fair correlation was found between the neutral lipid contents of the livers and the fat demonstrable histologically by scarlet R. There was further a correlation between the number of large hydropic cells and the non-glycogen water/PPN ratios. After administration of carbon tetrachloride this ratio increased whether or not hydropic cells were found. If, however, such cells were present, the ratio appeared to vary directly with their number (Fig. 14). The appearances of the cells in the periportal area before and after carbon tetrachloride injection showed the characteristic variations caused by the different protein contents of the diets (Kosterlitz, 1947). This is in agreement with the finding that carbon tetrachloride caused no major change in the relative concentrations of protein, phospholipin and nucleic acid (indices).

DISCUSSION.

This investigation was started in order to examine whether or not labile liver cytoplasm is broken down under the stress of carbon tetrachloride poisoning.

If this be so, then the livers of the rats fed on the 18 or 54 per cent casein diet ought to lose PPN and also show an increase in the nucleic acid indices (Kosterlitz, 1947). This was, however, not the case; on the contrary, on all diets, including the protein-free one, there was an increase in PPN and the indices remained almost constant. These findings not only indicate that carbon tetrachloride did not cause a breakdown of labile liver cytoplasm, at least not in the cells unaffected by necrosis and fatty degeneration; they also suggest that new cells were formed, the cytoplasm content of which was determined by the protein content of the diet. It would also appear from the increase in PPN, which was at its maximum on the first and second days after carbon tetrachloride, that the new cells were formed before the damaged cells had been removed, or that the number of new cells exceeded those whose cytoplasm had obviously been diminished. Whether the decrease in basophilia in the central area and its increase in the peripheral area of the lobule corresponded to changes in the ribonucleic acid content of the cells must be left an open question.

Simultaneously with the increase in PPN there was an increase in the non-glycogen water content of the liver and a rise in the ratio non-glycogen water/PPN. This rise in the ratio was present in all animals after administration of carbon tetrachloride, but reached particularly high values when large numbers of hydropic cells were present. The increase in water content was sometimes so pronounced that fluid oozed freely from the cut surface of the liver.

Similar increases in the protein and water content were found by Himsworth and Glynn (1945) in their work on the dietetic necrosis of the liver. These authors assumed that at least some of the extra protein and water was derived from absorbed blood plasma. It is unlikely that in our experiments this mechanism was responsible for the observed changes, since the protein/phospholipin ratio remained practically unchanged, and rat plasma contains much less phospholipin than liver tissue (Kosterlitz, 1947).

As far as the effects of diet on the degree of hepatic injury are concerned, our observations may be summarized thus: The hydropic and necrotic changes were most noticeable in the 18 per cent casein group, less in the 54 per cent casein group and almost absent in the protein-free group. If, however, the animals fed on the 54 per cent casein diet voluntarily restricted their food intake, very severe hydropic and necrotic changes took place. A pronounced loss of glycogen was observed on all diets. The quantity of fat found in the livers increased with the amount of casein in the diet. As restriction of food intake by itself reduces the fat content in the liver of rats on low choline diets (Best and Ridout, 1938), it is possible that the low content in neutral lipids in the protein-free group as compared with the 18 per cent casein may be largely explained by the low food intake of the former group (Table I). On the other hand, the highest lipid content was found in the 54 per cent casein group, the food intake of which was intermediate between the two other groups.

These results are in general agreement with the findings of Opie and Alford (1915), Davis and Whipple (1919), and Davis (1924), to which reference has already been made. Thus it would appear that in acute experiments and in the absence of protein depletion, which makes the liver very susceptible to this type of injury (Miller and Whipple, 1940, 1942), a maximum protective effect will be produced by a diet rich in carbohydrate and low in protein. In chronic experiments, diets rich in carbohydrate and adequate in protein will protect dogs and

rats from carbon tetrachloride poisoning (Bollman and Mann, 1936; Bollman, 1943), while low-protein diets increase the susceptibility to both chloroform and carbon tetrachloride (Goldschmidt *et al.*, 1939; György *et al.*, 1946).

SUMMARY.

1. Adult male rats were placed for three days on a protein-free, 18 per cent casein or 54 per cent casein diet and then injected with carbon tetrachloride.

2. The protein, phospholipin and nucleic acid contents of the livers were increased in all three diet groups. Since the protein/phospholipin and protein/nucleic acid ratios were not significantly altered, administration of carbon tetrachloride did not cause a breakdown of labile liver cytoplasm—at least not in the cells unaffected by necrosis and fatty degeneration. The observed changes are interpreted as representing the formation of new liver cells; the amount of labile cytoplasm present in these cells was determined by the protein content of the diet. This was also borne out by the histological appearance of the cells, which showed the characteristic variations caused by differences in the protein contents of the diets.

3. Correlations were found, first, between the neutral lipid content of the liver and the fat histologically demonstrable by scarlet R and, secondly, between the non-glycogen water content of the liver and the number of cells showing hydropic degeneration.

4. The liver damage as measured by central necrosis, hydropic degeneration and deposition of neutral lipids was least on the protein-free diet. The rats on the 18 per cent casein diet showed the highest incidence of hydropic degeneration, and those on the 54 per cent casein diet the greatest deposition of neutral lipids. On all diets there was a marked loss of glycogen. These results are in agreement with the findings of Opie and Alford (1915), Davis and Whipple (1919), and Davis (1924), viz., if given for short periods before the administration of chloroform or carbon tetrachloride, a diet rich in carbohydrate, low in fat and protein gives the highest protection against liver injury. On the other hand, prolonged dietary protein deficiency leads to protein depletion, which renders the liver highly susceptible to this type of injury (Miller and Whipple, 1940, 1942).

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THE DETECTION OF A PRODUCT OF THE BLOOD GROUP *O* GENE AND THE RELATIONSHIP OF THE SO-CALLED O-SUBSTANCE TO THE AGGLUTINOGENS A AND B.

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THE original classification of human bloods into four groups, A, B, AB and O, was based on the presence or absence of agglutinable substances A and B within the erythrocyte (Landsteiner, 1901; Decastello and Sturli, 1902; Jansky, 1907; Moss, 1910), group O erythrocytes being considered as cells devoid of the agglutinogens A and B, and recognized only by the absence of the A and B specific characters. Ottenberg and Epstein (1908) first suggested that the blood group character is inherited, and von Dungern and Hirszfeld (1910) subsequently confirmed this. They showed that the group property behaves according to Mendelian laws, and considered that the heredity of the A and B agglutinogens depended on two pairs of allelomorphic genes, A and a, representing the presence or absence of the A agglutinogen, and B and b controlling the appearance or absence of the agglutinogen B. Bernstein (1924) examined the available family data, and found that this did not agree with what would be expected on the basis of von Dungern and Hirszfeld's theory. Bernstein therefore brought forward a theory based on the existence of three allelomorphic genes *A*, *B* and *O*, and according to which the blood group of the individual depends on the presence of any two of the three genes. The occurrence of group O would then be due to the operation of a pair of *O* genes recessive in effect, whereas groups A and B would each be produced by the substitution of the corresponding genes at the locus of *O*. The dominance of gene *A* or *B* over *O* was considered by Bernstein to be complete, and the effect of the presence of a single gene *A* or *B* in the chromosome of the heterozygote would therefore be indistinguishable from that induced by the presence of a double gene-dose, *AA* or *BB*, as it occurs in the homozygote. If, however, the genes *A* and *B* are not dominant over the gene *O* one would expect the presence, even in heterozygous erythrocytes and tissue cells, of a

gene product O analogous to the agglutinogens A and B. In the homozygous OO red cells and tissues one would expect in either case the presence of the gene product O. The occurrence of the A and B agglutinogens in the erythrocytes is established by means of their agglutination with the iso-agglutinins anti-A and anti-B, which occur naturally in the blood serum of persons belonging to groups B and A respectively. Anti-O agglutinins, however, are found in human serum only very rarely and in special circumstances, and, therefore, means are generally not available for the detection of the agglutinogen should it occur in the red-cells of group O persons or in the cells of the heterozygotes AO and BO.

Landsteiner and Witt (1926) and Landsteiner and Levine (1926) showed that very infrequently the serum of persons belonging to group A_1 or A_1B contains an "irregular" isoagglutinin which reacts weakly with O cells. More recently an instance of iso-immunization of an A_1B person by O cells with the production of a weakly reacting anti-O iso-agglutinin has been reported by Wiener, Oremland, Hyman and Samwick (1941). Dockeray and Sachs (1941) describe a case of autohaemagglutination which was most probably due to the presence of an anti-O agglutinin in the patient's serum, and Henry (1946) reports a human anti-O agglutinin of unusual strength and discusses its place in the ABO blood group scheme.

The virtual absence of potent human anti-O agglutinins has resulted in an extensive search for animal sera possessing a similar property, and numerous attempts have been made to immunize various species with human O cells or O active secretions. Thus Hooker and Anderson (1921) produced an anti-O serum in a single rabbit by immunization with human O erythrocytes, and somewhat later Matta (1937) confirmed this observation. Schiff (1927) and Greenfield (1928) found that certain normal cattle sera after absorption with A_1B red cells agglutinated O red cells strongly, and this was the only reliable source of anti-O agglutinin until Eisler (1930, 1931) observed the formation of a powerful anti-O serum in a goat after immunization with *Shigella dysenteriae*. The serum was examined by Landsteiner and Levine (1931), Schiff (1934), and by Hirszfeld and Amzel (1940), who reported it to be a very useful reagent for the agglutination of O red cells and for the detection in inhibition tests of a so-called O-substance. More recently Morgan and Waddell (1945) produced without difficulty immune sera, which reacted strongly with O red cells, by the immunization of rabbits with a purified preparation of O-substance isolated from the pseudomucinous ovarian cyst fluid obtained from a person belonging to group O.

It was soon observed that these anti-O sera reacted strongly with red cells belonging to group A_2 and with certain group B red cells as well as with group O red cells, whereas other group B red cells and all A_1 red cells reacted weakly. As most of the anti-O sera used in these tests had been absorbed with A_1B red cells to render them free from anti-A, anti-B and species agglutinins it is not possible to comment on their activity with A_1B red cells, except to state that some A_1B red cells reacted after the serum had been thoroughly absorbed with a particular specimen of A_1B red cells.

The reaction of the anti-O sera with O erythrocytes was explained by Thomsen (1932) as being due to a specific O agglutinogen contained in the red cells, the O agglutinogen being considered a direct product of the activity of the *O* gene. In support of this explanation it was observed that the strongest reactions were

given by erythrocytes of the genotype OO, whereas most red cells of the genotype AB reacted scarcely at all. The irregular reactions of red cells belonging to the groups A and B were considered by Thomsen to be due to heterozygosity. The weaker reactions of A_1 and B erythrocytes were believed to be the result of the partial dominance of the gene A_1 and B over O; the stronger reactions of A_2 were considered to be due to heterozygosity reinforced by the less complete dominance of the gene A_2 over O. Thomsen's conclusions seemed to be supported by Sasaki (1932), who produced evidence that the O substance secreted in human saliva was not species specific in that powerful anti-human species sera were not inhibited in their action with the homologous antigen by saliva showing strong blood group activity.

Somewhat later, Hirszfeld (1934) suggested that the anti-O agglutinins are hetero-human species agglutinins, and considered that the greater the amount of the group agglutinogen on the red cell surface the less the amount of species agglutinogen present. Erythrocytes belonging to the group AB would in consequence react with the anti-serum weakly or not at all, whereas red cells possessing poorly developed agglutinogens only, such as A_2, would react strongly, and those devoid of both A and B agglutinogens, as in group O, would be most readily agglutinated.

A closer study of the action of rabbit anti-human serum and cattle anti-O serum on red cells of all genotypes was undertaken by Moureau (1935), who found that the cells were equally agglutinated by the rabbit anti-human species serum, and that such a serum was weakened to the same extent in its homologous reaction by absorption with red cells of all genotypes. The cattle serum, on the other hand, enabled a distinction to be recognized between the different red cells, and Moureau concluded that the antigen revealed by the cattle serum is not characteristic of the human species.

The result obtained by the use of cattle anti-O sera led several workers to conclude that this serum detected a substance, the presence of which was controlled by Bernstein's gene *O*, and the occurrence of which could be used to distinguish heterozygous from homozygous individuals belonging to groups A or B (Matta, 1937; Dahr, 1938, 1939).

The examination of the behaviour of a number of erythrocytes of authentic genotype with cattle anti-O serum convinced Moureau (1946) that the genotype of the red cells (AA, BB, AO, and BO) does not influence the serological agglutination or absorption reactions with the serum, and he concluded that this anti-O reagent does not detect a product of the Bernstein *O* gene. Moureau considers that Matta (1937), who, with Dahr (1938, 1939), believes it is possible to detect the heterozygous red cells, AO and BO, completely misinterpreted the agglutination reactions observed. According to Lambert (1941), Dahr's results do not carry any certain conviction of the possibility of determining the genotype of erythrocytes, and he is of the opinion that the amount of O element in human red cells decreases progressively from type O to AB—a conclusion in complete agreement with the views of Hirszfeld and Kostuch (1938).

Lambert (1941) studied further the agglutination of human red cells with Eisler's goat anti-O serum, and showed that the anti-O immune body is readily removed by *Shigella dysenteriae*, as is the anti-O component of normal cattle serum. The O component of human erythrocytes is therefore serologically related in some way to a substance in *Shigella dysenteriae*. The results of these

experiments, which establish the presence of a closely related specific substance in bacteria, and the occurrence of a specific anti-O serum in cattle, suggest that the so-called O component of human erythrocytes is a heterogenetic antigen, analogous to the heterogenetic antigen of Forssman, which itself shows a close relationship to the A agglutinogen of human red cells.

As indicated above, the reactivity of A and B red cells with the different so-called anti-O reagents has frequently been assumed to be due to the presence in the erythrocyte of an O substance, which is a product of the *O* gene, but the frequency of occurrence of erythrocytes which fail to react with anti-O serum, although similar to the expected number of homozygotes in the population, is found to bear no exact relation to it.

Recently there have become available to us a number of human sera which have the property of agglutinating O red cells strongly at room temperature, but which show no power to agglutinate red cells of the genotype A_1B. Boorman, Dodd and Gilbey (1948) have examined the behaviour of 500 blood samples with one of these anti-O sera, "Mrs. G." The results obtained were in very close agreement with the frequencies, calculated by the method of Stevens (1938), which would be expected if one were using an antibody detecting the presence of the Bernstein *O* gene. The serum was found to react with the agglutinogen O but never with the agglutinogen A_1 or B. It would appear, therefore, that the serum "Mrs. G." picks out the red cells containing the product of the *O* gene. The reactivity of the serum with A_2B red cells was demonstrated.

The serum "Mrs. G." thus provides a means whereby the nature of the so-called O-substances described in the literature can be determined, for it is evident that a substance which is a product of the *O* gene will inhibit the agglutinating action of the serum "Mrs. G." on O erythrocytes, whereas the other substances, misnamed O-substances on the basis of their reactivity with the so-called anti-O sera, will fail to cause inhibition. The results of inhibition and agglutination tests showed at once that the O-substances described by all previous workers are not related serologically to the serum "Mrs. G.," and are therefore not products of the *O* gene. To differentiate these substances from the true O-substance we designate them by the letter H, for reasons we shall discuss later. Similarly, sera which react strongly with O red cells but which are not serologically related to the serum "Mrs. G." are termed anti-H sera, and evidence is presented which demonstrates that probably all the so-called anti-O sera of animal origin are, in fact, anti-H sera. The behaviour of the different anti-O sera with saliva, cyst fluids, serologically active preparations obtained from O erythrocytes and purified preparations of the H-substance, are given in this paper, together with an account of a suggested relationship of this substance to the gene products A, B and O.

EXPERIMENTAL.

Material and Methods.

The serological reactions studied involved the use of preparations of the human blood-group-substances and of the A and H specific substances of pig gastric mucin. The methods for preparing these materials have already been given. Thus, human group A substance was prepared from pseudomucinous ovarian cyst-fluids (Morgan and van Heyningen, 1944) according to a modified

procedure based on the original method of Morgan and King (1943). The A-substance was devoid of H specificity and gave the following analytical figures: C 46·1, H 7·2, N 5·7 (Dumas), CH_3CO, 8·8 per cent, $[\alpha] + 16 \pm 3°$. The material was free from P and S, was electrophoretically homogeneous at pH 4·0 and 8·0, and sedimented as a single component in the Svedberg Ultracentrifuge (S_{20}, $5{\cdot}1 \times 10^{-13}$ uncorrected). Acid hydrolysis of the material gave rise to 36 per cent hexosamine, determined as glucosamine, and 57 per cent reducing sugars estimated as glucose. The H-substance was isolated and purified in a similar manner from the cyst fluid of "secretors" belonging to group O (Morgan and Waddell, 1945) and gave similar analytical figures. The specific rotation was $[\alpha]_{5461} - 20°$. The material was largely homogeneous when examined in the Tiselius electrophoresis apparatus, but a minor component with somewhat different properties was also present. Acid hydrolysis of the H-substance gave rise to 33 per cent hexosamine and about 50 per cent reduction. The pig mucin preparations were isolated as described by Morgan and King (1943) from carefully selected individual pig's stomach-linings and showed A or H specificity only (Bendich, Kabat and Bezer, 1946; Aminoff, Morgan and Watkins, 1946). The saliva specimens employed were collected and immediately heated at 100° for 10–15 minutes, and then used at once in inhibition tests or frozen at − 10° until required. Extracts of O erythrocytes were made by the method of Hallauer (1934), and subsequently purified by fractionation of the material from solution in water or organic solvents by alcohol.

The serological reagents used comprised: (1) An anti-serum prepared in the rabbit by immunization with the H-substance of human origin (Morgan and Waddell, 1945). (2) A naturally occurring anti-H cattle serum. Both sera were absorbed with fresh A_1B red cells from a donor (R.R.R.) until they no longer reacted with the absorbing red cells. Red cells from the same donor were used to check the reactivity of these anti-O reagents with A_1B red cells each time inhibition tests were performed. (3) Two human anti-O sera, "Mrs. G." and "Mrs. L.," were kindly supplied by Miss Boorman, Miss Dodd and Mr. Gilbey, of the National Blood Transfusion Service. A similar serum, "Mrs. H." (group AB), was given to us by Dr. L. Diamond, Boston, U.S.A. (4) A fourth human serum, "Mrs. C.," was obtained through the courtesy of Mr. Dunsford of the National Blood Transfusion Service, Sheffield. The anti-O cattle serum was incompletely absorbed with A_1B red cells from R.R.R., and gave some agglutination with A_1B red cells at 1/2 dilution; inhibition tests were therefore carried out with this serum at a dilution of 1/4. All tests using O red cells were carried out with freshly prepared cell suspensions, since red cell suspensions more than 2 or 3 hours old are less sensitive to agglutination by anti-O serum.

Owing to the small amounts of the human sera available the inhibition tests were mostly carried out according to the following simplified technique: One drop of test serum was added to one drop of the saliva, crude cyst preparation (1 per cent), the H-substance (1 per cent) or the material obtained from erythrocytes (1 per cent) and, after mixing and standing at room temperature for 30 minutes, the mixture was titrated in serial double dilution in the usual manner. One drop of the serum mixed with an equal volume of saline and titrated in the same way served as a control. One drop of a 0·5 per cent suspension of fresh erythrocytes was then added to all tubes, the contents thoroughly mixed, and allowed to stand at room temperature for 2 hours before reading. Neutralization

of the antibody by the preparation under test was indicated by an agglutination titre lower than that given by the control. Where possible the agglutination-inhibition test was performed by the more accurate method used earlier by Morgan and van Heyningen (1944). Results obtained when both tests were performed were qualitatively identical.

Results.

Five of the test-sera were titrated simultaneously with fresh red cells of different genotype. The results obtained with a few specimens only of red cells are set out in Table I, from which it will be seen that there was no significant

TABLE I.—*The Agglutination Reactions of Erythrocytes of Different Genotypes with Five Test-Reagents.*

Blood group of test cells.	Dilution of rabbit anti-H.*					Dilution of cattle anti-H.*					Dilution of human Anti-O† ("Mrs. G.").					
	1/4	1/8	1/16	1/32	1/64	1/4	1/8	1/16	1/32	1/64	1/2	1/4	1/8	1/16	1/32	1/64
O	3	3	2	1	1	3	3	3	2	1	3	3	2	2	1	0
O	3	3	3	2	1	3	3	3	2	1	4	3	3	2	2	1
A_2	3	3	2	1	0	3	3	2	1	0	4	3	3	2	1	1
B	2	1	0	0	0	2	1	0	0	0	—	—	—	—	—	—
BB	1	0	0	0	0	—	—	—	—	—	0	0	0	0	0	0
A_1O	2	1	0	0	0	1	1	0	0	0	3	3	2	1	1	0
A_1A_1	1	0	0	0	0	—	—	—	—	—	1	0	0	0	0	0
A_1B	0	0	0	0	0	0	0	0	0	0	—	—	—	—	—	—
A_2B	2	2	1	0	0	—	—	—	—	—	3	3	2	1	1	0

Blood group of test cells.	Dilution of human anti-O ("Mrs. H.")						Dilution of human anti-H ("Mrs. C.").†				
	1/2	1/4	1/8	1/16	1/32	1/64	1/2	1/4	1/8	1/16	1/32
O	3	3	3	3	2	1	3	3	2	2	1
O	3	3	3	2	2	1	3	3	2	2	1
A_2	3	3	3	2	2	1	3	3	2	1	0
B	3	2	2	1	0	0	—	—	—	—	—
BB	—	—	—	—	—	—	—	—	—	—	—
A_1O	3	2	2	1	0	0	2	1	0	0	0
A_1A_1	—	—	—	—	—	—	—	—	—	—	—
A_1B	0	0	0	0	0	0	—	—	—	—	—
A_2B	—	—	—	—	—	—	3	2	2	1	0

* Absorbed with A_1B cells.

† β-agglutinins removed.

Degrees of agglutination: 0, no agglutination; 1, a few groups of 2–3 cells; 2, larger groups with many free cells; 3, clumps visible to the naked eye.

qualitative difference in the reactivity to be observed. Erythrocytes of the genotype OO react most strongly, and certain A_2 red cells react nearly as well. The human serum "Mrs. H." agglutinated strongly the B and A_1 cells tested, but failed to agglutinate A_1B red cells. The presence of a strong β agglutinin in the serum "Mrs. G." necessitated its absorption with A_1B red cells or the addition of excess B-substance before it could be used to test the reactivity of red cells containing the B agglutinogen. The homozygous bloods A_1A_1 and BB were obtained from two children both of whose parents belonged to group A_1B. The A_1B red cells given in Table I were obtained from our "standard" donor (R.R.R.), and reacted but weakly with undiluted rabbit serum. A more correct impression of the degree of agglutination given by an immune rabbit

serum with A_1B red cells in general, after partial absorption of the serum with a specimen of weakly reacting A_1B red cells, is given by the agglutination results summarized in Table II where the reactivity of 74 unselected specimens of AB

TABLE II.—*The Agglutination Titre of A_1B and A_2B Cells with Immune Rabbit Anti-H Serum.*

Group of cells.	Number tested.	Number of specimens agglutinating to a titre of—					
		1/6	1/12	1/24	1/48	1/96	1/192
A_1B	65	4	18	24	13	1	1
A_2B	9	0	0	0	0	2	7

red cells is set out. It will be seen that most of the A_1B red cells reacted at a serum dilution of 1/24, one only agglutinated at a serum dilution above 1/96, whereas 4, including the red cells used for absorption, agglutinated at a serum dilution less than 1/12. On the assumption that the test serum at dilutions at or greater than 1/12 was free from α and β agglutinins and human species agglutinins, the variation in sensitivity recorded in Table II for the A_1B red cells may be accepted as a measure of the amount of substance on the erythrocyte surface which is specifically related to the antibody in the immune rabbit serum. The A_2B red cells reacted much more strongly than the A_1B red cells, and, although the number tested is small, the results indicate that A_2B red cells possess considerably more of the specific substance detected by immune rabbit serum than do A_1B red cells.

An examination of the salivas obtained from the same donors as were used to supply the erythrocytes for the agglutination reactions set out in Table I revealed at once that the sera were of two distinct kinds—those whose action on O red cells was completely inhibited by the saliva specimens, and those whose power to agglutinate O red cells was unimpaired in the presence of all the salivas

TABLE III.—*Showing the Activity of Different Saliva Specimens in Agglutination-Inhibition Tests with Four Different Test-sera.*

Salivas from donors belonging to group—	Dilution of "Mrs. G." serum.						Dilution of rabbit serum.				
	1/4	1/8	1/16	1/32	1/64	1/128	1/16	1/32	1/64	1/128	1/256
O	3	3	3	2	2	1	0	0	0	0	0
O	3	3	3	2	2	1	0	0	0	0	0
A_1	3	3	3	2	2	1	0	0	0	0	0
A_2	3	3	3	2	2	1	0	0	0	0	0
B	3	3	3	2	2	1	0	0	0	0	0
A_1B	3	3	3	2	2	1	0	0	0	0	0
O*	3	3	3	2	2	1	3	2	1	1	0
Saline	3	3	3	2	2	1	3	3	2	1	0

Salivas from donors belonging to group—	Dilution of "Mrs. C." serum.					Dilution of cattle serum.			
	1/4	1/8	1/16	1/32	1/64	1/8	1/16	1/32	1/64
O	0	0	0	0	0	0	0	0	0
O	0	0	0	0	0	0	0	0	0
A_1	0	0	0	0	0	0	0	0	0
A_2	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0
A_1B	0	0	0	0	0	0	0	0	0
O*	3	2	1	0	0	3	3	2	1
Saline	3	2	1	0	0	3	3	2	1

* Non-secretor.

examined. To the first group belonged the natural cattle anti-O serum, the immune rabbit serum and the human serum, "Mrs. C." The second group contained the human sera "Mrs. G.," "Mrs. H." and "Mrs. L." The saliva from a group O non-secretor was included, and was found to have no influence on the agglutination of O cells by any of the sera examined. The results of these tests are given in Table III.

The fact that the saliva specimens contained no substance capable of neutralizing the authentic anti-O serum "Mrs. G.", indicates that they do not contain O-substance. The saliva from a "secretor" of group A_1B which cannot contain the O-substance because the *O* gene is absent from the genotype inhibits completely, however, the agglutinating action of the natural cattle serum, the immune rabbit serum and the human serum "Mrs. C." It may be concluded, therefore, that the substance secreted in the saliva is not a true O-substance. Substances which inhibit the action of natural cattle, immune rabbit and the human serum "Mrs. C." are termed H-substances, and these sera are anti-H sera. H-substance is strictly specific in its reactions with H antibody and is without influence on O antibody.

The inhibition tests were then repeated using 0·5 per cent solutions of freeze-dried ovarian cyst fluids. Eleven were obtained from persons who were known to be strong secretors, and five were from non-secretors of material inhibiting rabbit anti-H serum. Again the agglutinating action of the serum "Mrs. G." on O red cells was not inhibited by the majority of the cyst fluids, yet the fluids, when obtained from "secretors," inhibited completely the agglutinating action of the immune rabbit serum. The fluids therefore contained large amounts of H-substance irrespective of the blood-group of the patient from whom they were obtained. The amount of H-substance in cyst fluids obtained from persons belonging to group O, however, was many times greater than in the cyst fluids from group A persons. It was observed that cyst fluids 60 and 87 gave titration end-points one tube lower than in the control series in which the serum "Mrs. G." was titrated in saline in place of cyst substance, and cyst fluids 91 and 97 showed stronger inhibition, the latter having an inhibition index of 16. A test employing a purified H-substance prepared from cyst 61 confirmed that this material still contained a substance which would neutralize the anti-O agglutinin in the serum "Mrs. G." The partial inhibition obtained indicated that the fluids contained a small amount only of a true O-substance. The results of the inhibition tests are set out in Table IV. The purified H-substance obtained from cyst 61 was separated into five fractions by precipitating the material from formamide solution by alcohol, but no substance showing greater power than the starting material to inhibit the serum "Mrs. G." was encountered. The extracts of O erythrocytes inhibited strongly the agglutinating action of the serum "Mrs. G.," and it will be seen that the material contained H-substance as well as O-substance, but this is to be expected, as both substances are known to be components of the red cell membrane, as indicated by the reactivity of the red cells with anti-H and anti-O sera (see Table I). The isolation and characterization of the O-substance from erythrocytes and cyst fluids will be reported later.

In view of the fact that the activity of the H-substance isolated from pig gastric mucin had been determined against the rabbit anti-H immune serum only, it was of considerable interest to determine whether the material con-

TABLE IV.—*The Inhibition of the Agglutination of O Cells by* (a) *the Serum "Mrs. G." and* (b) *Immune Rabbit Serum by Ovarian Cyst Fluids and other Substances.*

Substance added.	Blood group of donor.	Dilution of serum giving agglutination. "Mrs. G." 1/8	1/16	1/32	1/64	1/128	1/256	Rabbit anti-H. 1/16	1/32	1/64	1/128
Crude cyst fluid :											
No. 55	O	3	3	2	2	1	0	0	0	0	0
„ 61	O	3	3	2	1	0	0	0	0	0	0
„ 68*	O	3	3	3	2	1	0	3	3	2	1
„ 72	O	3	3	2	1	1	0	0	0	0	0
„ 74	O	3	3	3	2	1	0	0	0	0	0
„ 87*	O	3	2	1	1	0	0	3	3	2	1
„ 91	O	3	2	1	0	0	0	0	0	0	0
„ 62	A_2	3	3	3	2	1	0	3	3	2	1
„ 49*	A	3	3	3	2	1	0	3	3	2	1
„ 58	A	3	3	3	2	1	0	0	0	0	0
„ 73	A	3	3	3	2	1	0	0	0	0	0
„ 75*	A_1	3	3	3	2	1	0	3	2	1	0
„ 78	A_1	3	3	2	2	1	0	0	0	0	0
„ 82	A_1	3	3	2	2	1	0	0	0	0	0
„ 95	A_1	3	3	3	2	1	0	0	0	0	0
„ 97*	A	2	1	0	0	0	0	3	2	1	0
Purified 61/28 (H-substance)	O	3	2	1	0	0	0	0	0	0	0
Crude O-substance from O red cells	O	0	0	0	0	0	0	0	0	0	0
Purified human A-substance	A_1	3	3	3	2	1	0	3	3	2	1
Hog mucin :											
H-substance	..	3	3	3	2	1	0	0	0	0	0
A-substance	..	3	3	3	2	1	0	3	3	2	1
Saline	..	3	3	3	2	1	0	3	3	2	1

* Non-secretor according to inhibition test with rabbit anti-H serum.

tained any O-substance. Similarly, a specimen of our purest human A-substance and a preparation of pig mucin A-substance which had been isolated from a single stomach and showed no power to inhibit cattle and rabbit sera were examined at the same time. The preparations were without action on the anti-O sera "Mrs. G." and "Mrs. H."

Schiff (1934) observed that whereas the titre of an immune goat anti-H serum prepared against *Shigella dysenteriae* was lowered by absorption with a culture of *Shigella dysenteriae*, that of human anti-O serum was not so reduced. The action of *Shigella dysenteriae* on three of the anti-sera used in our investigations and on anti-B serum was therefore studied. A thick suspension of bacilli in 0·5 per cent formalin was measured (0·25 ml.) into four tubes and centrifuged. The supernatant fluid was discarded, and to the deposit of organisms in each tube 0·2 ml. of the appropriate serum was added and the bacilli were re-suspended by vigorous stirring. The absorption of anti-B (β) agglutinins by the *Shigella dysenteriae* was tested by the addition of B red cells in place of O red cells, and any lowering of the agglutination titre observed, used as a measure of non-specific absorption. The results are shown in Table V and establish that, whereas the serum "Mrs. G." is uninfluenced in its power to agglutinate O red cells by treatment with a thick suspension of *Shigella dysenteriae*, the human serum "Mrs. C." is exhausted of anti-H agglutinin (so-called anti-O agglutinin), as is the rabbit serum. There was no significant decrease in β agglutinins in the

TABLE V.—*The Effect of Treatment of Sera with Anti-H and Anti-O Specificity with a Suspension of* Shigella dysenteriae.

Serum.	Treatment.	Dilution of serum.						
		1/2	1/4	1/8	1/16	1/32	1/64	1/128
Anti-O (" Mrs. G.")	Unabsorbed	3	3	3	3	3	2	1
	Absorbed	3	3	3	3	2	2	1
Anti-H (" Mrs. C.")	Unabsorbed	3	3	2	2	1	0	0
	Absorbed	1	0	0	0	0	0	0
Rabbit Anti-H	Unabsorbed	4	3	3	3	2	2	1
	Absorbed	2	1	0	0	0	0	0
Control :								
Anti-B (human)	Unabsorbed	4	4	3	2	2	1	0
	Absorbed	4	4	3	2	1	1	0

human anti-B serum when exposed to a similar concentration of the same culture of *Shigella dysenteriae*, and it may be concluded therefore that the reduction in the so-called O agglutinin observed with the serum " Mrs. C." and the rabbit serum is specific in nature. It would appear that the differentiation of human anti-O sera into those which detect a product of the *O* gene and those which react with the H character can be accomplished by means of absorption with *Shigella dysenteriae*.

Since this work was completed other sera agglutinating O red cells have been received and examined. Two human sera, " Mrs. R." and " Mrs. B.," were found to be similar to the serum "Mrs. C.," and are therefore anti-H sera. A specimen of anti-serum produced in the goat by immunization with *Shigella dysenteriae* was kindly sent by Dr. E. A. Kabat, of New York, and proved to be anti-H in character, thus supporting our belief that the majority of so-called anti-O sera of animal origin are anti-H sera. This is not to say that anti-O sera specific for a product of the *O* gene cannot be produced in animals, but that anti-H sera being heterogenetic in character are likely to be encountered more frequently.

DISCUSSION.

Until 1927 no antibody, either natural or immune, was known which would react in an apparently specific manner with O erythrocytes, but in that year Schiff (1927), and somewhat later Greenfield (1928), demonstrated that certain carefully selected and absorbed normal cattle-sera gave rise to the agglutination of O red cells, but were without significant action on most erythrocytes of the genotype AB. Soon after this discovery it was observed that certain persons belonging to group O secreted a substance in their saliva which was able to neutralize the agglutinating action of the absorbed cattle serum on O-red cells, and from this time a serum with these characters was called an anti-O serum. A soluble substance which neutralized the agglutinating action of the serum on O red cells was termed O-substance. Is the so-called O-substance a true product of the *O* gene, or is this substance unrelated to the A and B substances and a basic or primary material quite distinct from the ABO group specific substances, as was suggested by Witebsky and Klendshoj (1941) ?

The strongest evidence that the so-called O-substance is not a product of the *O* gene is based on the fact that individuals of genotype A_1B who lack the *O* gene, and who secrete their blood group substances (A and B) in a water-soluble

form, also secrete this so-called O-substance. What, then, is the explanation for the presence of this factor in the secretions ?

The detection of the so-called O-substance in saliva by Sasaki (1932), in gastric juice by Witebsky and Klendshoj (1941), and in pseudomucinous ovarian cyst-fluids by Morgan and van Heyningen (1944), and its characterization as a mucoid very similar in chemical properties to the A and B substances (Morgan and Waddell, 1945), make it expedient to consider carefully the relationship of this substance to the A and B specific substances. Furthermore it is desirable, in view of the widespread designation of the material as O-substance and frequent assumption that it is a product of the *O* gene, to obtain evidence which will establish its exact serological character.

Up to the present time no direct evidence for the existence of a product of the *O* gene has been brought forward, largely because serological reagents have not been available whereby the O-substance could be detected with certainty. An approach having for its objective the detection of the product of the *O* gene, and based on the results of agglutination or inhibition tests, must assume that O red cells contain a product of this gene. The characterization by Boorman, Dodd and Gilbey (1948) of the serum " Mrs. G." as a reagent which detects the product of the *O* gene provides means whereby the nature of the so-called O-substance can be investigated.

Agglutination and inhibition tests have been carried out with the serum " Mrs. G." and two other similar sera, " Mrs. H." and " Mrs. L.," and it is evident that these sera behave quite differently from the cattle and immune rabbit and goat sera and the sera " Mrs. C.," " Mrs. R." and " Mrs. B.," for whereas all members of the last group of sera are inhibited completely in their action on O red cells by the so-called O-substance present in the tissue fluids and secretions of all " secretors " irrespective of their blood group, the sera " Mrs. G.," " Mrs. H." and " Mrs. L." are not so inhibited. These observations would seem to establish that the O-substance described throughout the literature is not a product of the *O* gene. In order to avoid confusion, therefore, the substance might be better called H-substance in place of O-substance, as this would differentiate it as a heterogenetic, basic or primary substance common to the great majority of red cells, irrespective of their A B O group. Furthermore, the designation H-substance would serve to indicate the ubiquitous nature of the material, to emphasize that it is not a product of the *O* gene, and to establish that it is immunologically distinct from the substance on the erythrocyte surface which reacts with true anti-O sera. The so-called anti-O reagents such as cattle, goat and rabbit sera and the human sera " Mrs. C.," " Mrs. R." and " Mrs. B.," are, therefore, anti-H sera. Anti-O sera should now be classified as anti-O reagents only if they detect a product of the *O* gene and are uninfluenced in their action on O red cells by the H-substance.

In a few instances the anti-O serum, " Mrs. G.," was inhibited by some specimens of ovarian cyst fluid, thus indicating that a soluble substance, corresponding in specificity to O antibody, exists. Our experience leads us to believe that the true O substance is most probably secreted in a water-soluble form infrequently and weakly as compared with the products of the allelomorphic genes *A* and *B*, but we believe that the examination of a larger number of specimens of tissue fluids and secretions will reveal a more potent specimen of O-substance than has as yet been encountered. Quite potent preparations of

O-substance can be obtained from O red cells. It may be anticipated that a material devoid of H specificity and possessing alone the serological character O will ultimately be obtained from the red cells and secretions of persons who possess completely mutated genes, such as "Mrs. C.," "Mrs. B." and "Mrs. R." The results of immunizing man and animals with these materials will be of considerable interest in view of the difficulty of finding potent anti-O sera suitable for blood grouping studies. It is to be noted that persons possessing a serum antibody of the type anti-H would, if the antibody were active at 37°, be liable to a haemolytic reaction if transfused with blood cells rich in H-substance, as are the great majority of group O bloods.

Now that the existence of a true product of the *O* gene is demonstrated, and it may be accepted that the gene *O* is co-dominant with the allelomorphic genes *A* and *B*, the relationship of the H-substance to the A, B and O substances can be considered. Are they quite unrelated? This seems improbable in view of the fact that erythrocytes agglutinated strongly by anti-A or anti-B sera give weak reactions with anti-H serum, and red cells reacting weakly with anti-A or anti-B serum react strongly with anti-H serum. This is indeed the reciprocal relationship demonstrated by Hirszfeld and Amzel (1940), who considered the agglutinability by anti-A and anti-H (called anti-O) serum is an additive property, the sum of which is constant. The less H-substance the erythrocytes possess the more A-substance they contain. Further evidence that the H-substance is related in some way to the blood group substances can be found in the observation that all A_2 and A_2B bloods react with anti-H serum, which suggests that the gene forms responsible for the production of erythrocytes with these characters are partially mutated and transitional forms between *H* and *A*. Thus, a relationship between the H-substance and the A B O group substances seems to exist on the basis of the serological evidence.

All the evidence available at the present time indicates that the A and B specific group substances and the H-substance possess very similar chemical properties (Morgan, 1947). The A and H substances, for example, materials which have been carefully studied, have the same chemical composition in terms of C, H, N and acetyl analysis. After acid hydrolysis they both give rise to approximately the same amount of hexosamine and reducing sugars and the amino N and amino acid N are probably identical. The substances give rise to a maximum colour with Ehrlich's reagent, equivalent to about 12 per cent of N-acetylglucosamine, after they have been treated with dilute Na_2CO_3 at 100° for a few minutes. Treatment of both substances with 0·01 M HIO_4 causes complete destruction of the specific activities in a few hours at 20°, and neither substance shows a significant ultra-violet absorption within the range 220–270 mμ. Finally, no gross difference with respect to the number and quantity of the amino-acids contained in the A and H substances can be demonstrated by means of sensitive chromatographic methods. The group B substance has been less thoroughly investigated, but appears to behave similarly where the appropriate properties have been examined. It may be accepted, therefore, that the group substances and the H-substance have many properties in common, and since some of these properties are somewhat unique in character, it is reasonable to believe that these materials have a common structure differing only in the presence or absence of small chemical groups or in the sterochemical configuration of certain groups common to both substances. The close chemical similarity of the A and

H-substances would seem to indicate they are not unrelated. Differences, however, are recorded for the optical rotation of the A- and H-substances, and different enzymes are necessary for their serological inactivation (Morgan, 1946).

The experimental observations reported in this paper allow a modification of Hirszfeld's theory (Hirszfeld, 1947) to be proposed which would account for the presence of the H-substance, and would explain the reactivity of the erythrocytes with the two kinds of sera, anti-O and anti-H, which are known to give similar agglutination reactions with the majority of red cells. According to the modified scheme the genes *A*, *B* and *O* are formed as the result of mutations from a basic or primary gene *H*, which engenders the formation of the basic or primary substance H which is a precursor of the A, B and O substances. Between the pure *A*, *B* and *O* gene forms and the unmutated *H* form there are a series of transitional forms giving rise to varying amounts of A and H, B and H and O and H substances on the surface of the A, B and O erythrocytes respectively. This hypothesis implies that when mutation is complete, gene forms designated A_c, B_c and O_c arise which are devoid of gene *H*. It is in the serum of a person possessing completely mutated genes that one would expect to find an H antibody. It has been found in the serum of "Mrs. C." (genotype AO), and as anticipated the erythrocytes of this person failed to react with anti-H sera but agglutinated strongly with anti-O serum. The H-substance is present in the great majority of red cells, in fact in all red cells except those arising from the rare gene combinations, A_cO_c, A_cA_c, B_cB_c, B_cO_c, A_cB_c and O_cO_c, in which mutation of both genes from the basic gene is complete. An antibody to the H-substance, as encountered in the human sera "Mrs. C.," "Mrs. R." and "Mrs. B." and in the cattle and rabbit anti-H sera will agglutinate, therefore, the majority of red cells irrespective of their homozygous or heterozygous nature. On the assumption that all mutations have advanced beyond the H form, all group O red cells and hyterozygote AO and BO red cells will contain the O-substance, and therefore an anti-serum to this substance may be expected to agglutinate all red cells except those of group A_1B or homozygous A_1A_1 or BB red cells. These are precisely the reactions reported by Boorman, Dodd and Gilbey (1948) and recorded by us. Furthermore, it will be evident that the relationship of the H and O substance implied in the modification of Hirszfeld's theory agrees with the results reported concerning the neutralizing action of the so-called "O" substance of animal and human origin on the two kinds of anti-O sera. Thus it has been shown that the materials isolated from certain pig stomachs, from ovarian cyst fluids and salivas of "secretors," irrespective of their blood groups, all give rise to inhibition of the anti-H serum, but fail to inhibit, except weakly and in a few instances, the agglutination of O erythrocytes by the anti-O sera "Mrs. G.," "Mrs. L." and "Mrs. H."

The anti-O serum, "Mrs. G.," reacts with the agglutinogen A_2 as well as with the agglutinogen O, and thus confirms the suggestion made by Wiener (1944) that anti-O sera react with the properties determined by genes *O* and A_2. Wiener's explanation for the lack of agreement by earlier workers on the reactivity of O and A_2 red cells is probably incorrect, since this has not necessarily anything to do with the potency of the test reagents as he suggests, but depends on their specificity. Most O and A_2 red cells are agglutinated by the so-called anti-"O" reagents, which are in fact anti-H sera, by virtue of their content of H-substance.

There is no suggestion that the modification of Hirszfeld's theory given

above will explain all the known facts. It does explain satisfactorily the reactions of all erythrocytes and active secretions with the two kinds of anti-O sera. The ideas have already been extended (Boorman *et al.*, 1948) in an attempt to explain the reaction of the anti-O serum with the agglutinogen A_2. Two linked genes are postulated, and instead of three distinct mutations arising from the primary gene *H*, only two, *H* to *O* and *H* to *B*, are envisaged. At some stage in the change *H* to *O*, one of the two linked genes *OO* on the chromosome undergoes a mutation to *AO*, giving rise to the gene complex A_2. The other gene in the combination *AO* may mutate, giving rise to AA_1, which is the gene complex A_1, and may finally assume the gene form A_cA_{1c}. It is at once apparent that sub-group A_2 has two antibodies, anti-A (α) and anti-O, and sub-group A_1 two antibodies, anti-A (α) and anti-A_1 (α_1), as in fact they are known to have. The scheme also accounts for the qualitative difference, which undoubtedly exists, between the agglutinogen A_1 and A_2. The different nature of the two *A* genes arising as a result of the mutation of the double gene complex *OO* first to *AO* and then to AA_1 is probably due to sterochemical influence, as discussed earlier (Morgan, 1937) in connection with the formation of antibodies.

SUMMARY.

1. A number of human anti-O sera are described, the specificity of which characterize them as reacting with a product of the *O* gene. They are not inhibited in their action on O red cells by the so-called O-substance.

2. A water-soluble substance which inhibits the action of these human anti-O sera on O red cells has been obtained from human ovarian cyst fluids and erythrocytes. The substance, which is secreted infrequently, is believed to be a product of the *O* gene.

3. Evidence that the so-called O-substance is a basic material heterogenetic in character is given. This substance is designated by the symbol H.

4. Most of the so-called anti-O sera of animal origin are anti-H sera, and are not specific for a product of the *O* gene. Human anti-H sera are described.

5. A suggested relationship of the H-substance to the A, B and O agglutinogens is discussed.

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THE FIBRIN BARRIER REGARDED AS A FILTER IN INFLAMMATION.

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IT was shown previously (Hughes, 1945) that in strictly localized infections with a variety of organisms, the anti-bacterial immunity of the body was very little stimulated. It was suggested that this could be due to the fibrin in the zone of inflammation around the focus acting as a selective membrane, and allowing certain toxins to pass through but holding back the larger particles, such as bacteria themselves. It appeared desirable to attempt to discover whether fibrin acted as a selective filter and whether antigens would pass through it.

There are already clinical and *in vivo* observations giving an indication of the absorption that takes place from a focus. Fleming (1939) and Hite, Banks and Dack (1938) showed that there was little antihaemolysin response to natural staphylococcal infections. The latter authors also demonstrated that both in natural and in artificial sinuses there was poor absorption of typhoid vaccine, horse plasma or botulinus antitoxin. Menkin (1936, 1940), in his studies of inflammation, also offers evidence on the vital dyes and other colloidal or particulate substances. The composition of the inflammatory exudates themselves suggests that there is some selective holding back of the globulin.

The experiments which follow provide data for comparing these *in vivo* results with those obtained by direct filtration through fibrin filters.

FILTRATION METHOD.

Filters were prepared from citrated plasma by the addition of calcium chloride and thrombin. A 20 ml. volume of plasma with 5 ml. of 1 per cent calcium chloride and 50 units of thrombin was poured into a petri dish, 6 cm. in diameter, and allowed to clot and to retract in an incubator. It was then placed between layers of filter-paper and perforated zinc and pressed by a weight of 30 g. Larger membranes were made in a similar way. The fibrin membrane was supported on a coarse " gradocol " collodion membrane, average pore size between 0·6 and 0·9 μ. The gradocol membranes of this size, alone, will hold back bacteria, but there is no interference by them when the range of substances used in these experiments are filtered.

Graduation of the filters by the methods used for the gradocol membranes has not so far been satisfactory, since fibrin is too easily stretched and since it must be kept in isotonic solutions as, on transference to water, there is an increase in porosity ; for this latter reason all experiments were made with isotonic solutions. It is hoped that the technical difficulties may be overcome, and a mathematical assessment of the porosity obtained and published subsequently.

Characteristics of the filters.

The unstretched, supported, wet membrane retains its properties for several days and filtration can be stopped and recommenced at intervals, provided that no drying has taken place. Plasma, at first, gives good clot retraction, but this is progressively lost. The porosity of the membranes appears to be directly related to the degree of clot retraction, and plasma not more than a few hours old must be used.

Rate of flow.

No filtration was obtained without the help of diminished pressure. Using a water pump and an ordinary domestic water supply, solutions containing no proteins could be filtered at the rate of from 0·5 to 1·5 ml./hour. Where proteins were present in the fluids, as in filtering undiluted sera, the rate of flow was reduced until it was necessary to wait as much as eleven hours to obtain 1·0 ml. of filtrate. Within the fibrin filter and the supporting gradocol membrane some fluid is held and it is desirable to discard the first 1·0 ml. of filtrate ; after that constant samples can usually be obtained.

It became clear as the work proceeded that there were three main causes leading to variations in the results: first, differences in the freshness of the samples of plasma, leading to variations in clot retraction; second, differences in the salt concentration of the fluid being filtered; and third, differences in the filtration pressure. When these factors have been controlled remarkably constant results can be obtained.

Table I shows representative results when a negative pressure of 600–680 mm. Hg was used. The duration of each experiment was from 24 to 72 hours. It will be seen that clear-cut results are given by the vital dyes. Trypan blue is

TABLE I.—*Filtration through Supported Fibrin Membranes at a Pressure of 600 mm. Hg.*

Substance filtered.	Result.	Comments.
Vital dyes in Ringer's solution.		
Vital new red	Passed	This dye passes all fibrin filters at all times.
Trypan blue	Retained	All perfect filters retain this dye. With the least stretching of the filter or failure of clot retraction it will be allowed to pass.
Dahlia violet	As for vital new red	..
Aniline blue, Nile blue, Janus green, Gentian violet, Congo red, Chlorazol black E, Nigrosin	As for trypan blue	This result could depend in part on the size of the particles but also partly on the electrical charge.
Proteins.		
Diluted plasma 1%	Passed	..
Diluted serum 1%	Passed	..
Diluted globulin 1%	Passed	..
Undiluted serum	Concentration above filter. Filtrate shows reversal of albumin-globulin ratio	Fuller details in Table II.
Cl. welchii antitoxin diluted in Ringer	Concentration above filter, and dilute filtrate ½ original strength below	Antilecithinase activity.
Diphtheria antitoxin (undiluted human serum). Original strength 10 units	Slight concentration	Titrated on skin of rabbit.
Staphylococcus antihaemolysin	No demonstrable increase above filter or decrease below	In all these cases slight concentration would be undetected in titration by the serial dilution methods employed.
Haemagglutinins against group B cells	Passed	
Flagellar agglutinins against *B. proteus*	Passed	
Bacterial toxins.		
Diphtheria toxin	Passed	..
Cl. welchii toxin	Passed	..
Staphylococcus toxin	Concentrated above and diluted filtrate below	Even at these pressures no toxin can be detected for some hours, but eventually it appears in the filtrate.

apparently of such a size that it is just held back by perfect filters, but is allowed to pass by filters made from plasma that has been stored or by stretched filters. With filters supported only by the perforated metal discs supplied for use with gradocol membranes, there is bulging of the fibrin through the holes in the disc forming hemispherical blisters in which the pore size is much larger than in the unstretched membrane. This difficulty can be overcome if the fibrin is supported with a coarse gradocol membrane between it and the metal disc.

The results in filtering proteins were more difficult to interpret at this stage. No concentration of these substances could be demonstrated when a 1 per cent solution in Ringer or physiological saline was used, yet when undiluted sera were used there was definite concentration of the globulins. It is difficult to be sure that no concentration has taken place in these experiments. The ordinary methods of doubling and trebling dilutions miss minor variations and, also, these very slow experimental filtrations, under reduced pressure, have the disadvantage that the filtrate is concentrated by evaporation as it collects in drops on the filter and as it runs down the sides of the container, so an unfairly high figure is given for it.

Table II gives details of a filtration experiment with undiluted serum.

TABLE II.—*Filtration of Undiluted Serum.*

Serum	Total protein 7·25 g./100 ml.
Residue	Total protein 8·45 g./100 ml.
Filtrate	Total protein 3·65 g./100 ml.

Rate of flow 1 c.c. in 9 hours.

When undiluted serum or plasma is used the flow is impeded, the filtrate is more dilute than the original material and an alteration of the albumin-globulin ratio takes place.

It is interesting to compare these figures with the analysis of exudates published by various authors. McIver (1933) found blister fluid with a total protein of 3·7 gm. per 100 ml. Harkins (1942) gives 3·4 g. and 3·0 g. for two samples.

In Table III a more detailed analysis of one such filtration experiment is

TABLE III.

Material.	Total protein.	Albumin.	Globulin.	A/G ratio.
Serum . .	7·2 g./100 ml. .	5·6 g./100 ml. .	*1·6 g./100 ml. .	3·5/1
Residue . .	8·2 g./100 ml. .	6·2 g./100 ml. .	*2·0 g./100 ml. .	3/1
Filtrate . .	1·75 g./100 ml. .	1·6 g./100 ml. .	†0·15 g./100 ml. .	11/1

* By subtraction only.
† By direct estimation as well as subtraction.
Rate of flow 1 c.c. in 11 hours.

given. The method of estimation used was a modification of Fine's method given by Harrison (1939). All samples were duplicated; three colorimeter readings were taken, the cups reversed and three further readings taken on each, giving each figure in the table as the mean of twelve readings. Except in the case of the filtrate, the figures for the globulins were obtained by direct estimation of the precipitate as well as by subtraction of the figures for the albumin from the total proteins; in all cases the agreement was within 0·1 g.

per 100 ml. With the filtrate the actual amount present was so small that accurate direct estimation was impracticable.

The definite holding back of a proportion of the globulins in this experiment raised the hope that it might be possible to show that the passage of antitoxins was obstructed, but this had not proved possible at this pressure. In no case has there been a qualitative effect demonstrated and, in the quantitative experiments, though some concentration above the filter and diminution below was seen, the results are not striking.

It was pointed out earlier that diminished pressure was necessary to obtain a flow through the fibrin membrane. The pumps employed gave intermittent pressures up to 720 mm. Hg, but were usually about 500 mm. It was found in working with staphylococcus antihaemolysin (which appears to me very important in these experiments, and is probably about the critical size for these filters) that very variable results could be obtained if different filtration pressures were used. These results varied from complete holding back of the toxin to its passage, with little demonstrable concentration above the filter or dilution below; in most experiments the strength below the filter was about one-half that of the control.

Results obtained by filtration through fibrin can be compared with those obtained by studying absorption in the body only if the pressures are similar in both cases and it is necessary to consider the pressure in a lesion.

While filtration may take place during the early stages in the formation of an effusion the pressure will be low, for in no case can it be above that of the blood; a probable figure might be about 80–100 mm. Hg. This is much lower than the pressure necessary to produce a flow of fluid *in vitro* so, while those substances held back in these experiments will also be held back in the body, other substances such as staphylococcus toxin, which are merely impeded at a pressure of 500–720 mm. Hg, the ranges used here, may be entirely excluded at lower pressures.

The influence of the osmotic pressure was considered, and experiments done to estimate it when fibrin was the membrane. The osmotic pressure is low, due to the fact that all crystalloids will pass and that albumin is not held back. The total value appears to be only 20 mm. water after 72 hours or more when serum is dialysed against saline or Ringer and must be due mainly to the globulin fraction.

Whatever the conditions may be at the onset of inflammation and the formation of an exudate, sooner or later filtration will cease and the pressure on the two sides of the pyogenic membrane will be equalized. This is the state of affairs for most of the duration of an inflammatory effusion, and any exchange between the circulation and the inside of the lesion will be by diffusion only. This consideration led to some of the experiments being repeated, using diffusion across fibrin in place of filtration. I consider that these results are more likely to give a true picture of the process in the body.

DIFFUSION METHOD.

A miniature container was used consisting of two flanged glass cups (Fig. 1). Into the lower cup A, saline or Ringer's solution was put; across this a fibrin membrane was placed without stretching (it may be supported on a coarse

gradocol membrane). The upper portion, B, was placed above and held there by small spring paper clips. The fluid to be tested was introduced with a pipette through the hole in the stem of B.

The filter can be left for several days. In collecting the fluid it is necessary to withdraw it from B with a pipette and then to wash out quickly before separating the two parts, so that any contamination of the fluid in A may be with water only. The results are given in Table IV.

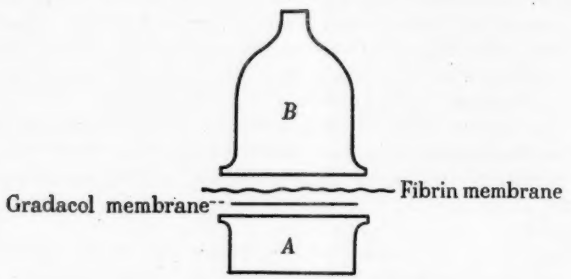

FIG. 1.—Miniature container for testing diffusion through fibrin.

TABLE IV.—*Dialysis Across a Fibrin Filter.*

Trypan blue	Held back	..
Vital new red	Passed	..
Staphylococcus toxin	Above filter	Titre 1/32.
	Control	1/32.
	Below filter	below ½ (none detected).
Diphtheria toxin	Above filter	Lf. 18.
	Control	18.
	Below filter	None detected.
Serum	Above filter	Total proteins : 7·75 g./100 ml.
	Below filter	Total proteins : 0·5 gm. Globulin : Too little to estimate trace present.
Diphtheria antitoxin (commercial purified)	Above filter	6,600 units/ml.
	Below filter after 17 hours	600 units/ml.

Before, however, we consider that we have reproduced the conditions in the wall of an inflammatory lesion, we have to ask if the pus cell will pass through these membranes as it would the natural ones in the body. If polymorphs will not pass, then we have a clot too dense or differing in some other way from the pyogenic membrane. The question was settled in the following way : a slide was taken and a drop of a broth culture of staphylococcus was dried on it. Over the slide a fibrin membrane was placed, so arranged that its edges extended beyond the slide. Fresh blood was placed on the membrane and allowed to clot. The slide was now incubated in a moist chamber. For a few hours no polymorphs could be demonstrated adhering to the glass below the membrane but, with over-night incubation, many of them were found to have passed through.

DISCUSSION.

Comparing these results with those obtained in the body it is desirable to consider to what extent the conditions approximate to those in an actual lesion. Precautions were taken to obtain full clot retraction, but it was necessary, for ease of manipulation, to use some pressure, and it may be thought that this would consolidate the structure of the clot. About 30 g. was the total weight used, and such a small pressure is unlikely to have much effect. Had the membranes been mounted without blotting they would still have been exposed to much higher pressures. After unpressed membranes had been used they were not found to differ from the pressed filters in any respect.

The problem of the right filtration pressure appears most important. While certain substances, such as the dye vital new red, will always pass through, and others, such as trypan blue, will be held back by all unstretched, perfect filters at all pressures, most of the interesting substances from the point of view of the immunologist are of such a size that under one set of conditions they will pass through while, under different conditions, they will be concentrated or even completely held back.

Now, most of these filtrations were made at a range of pressures between 500 and 680 mm. Hg, as at lower pressures little or no filtrate could be obtained. It is probable that the actual pressures in the body can never be much above 100 mm. Hg, and such a pressure will rapidly be neutralized by the formation of the exudates. In the body the pressure will act in one direction only, and will assist the passage of the serum proteins into the lesion and hamper that of the toxins into the circulation. When the pressure has been equalized and diffusion is the method by which substances pass, then fibrin forms an important barrier, and alone would account for a number of the observations made by other workers.

It would explain Menkin's (1936) observations on trypan blue in inflammation, as he believes. Bacterial bodies and a proportion of their toxins will be held back once the barrier is established, but there will be some seepage of proteins at every stage and polymorphs will be able to pass into the lesion.

It is interesting to note how good an imitation of the composition of an exudate can be obtained by filtering serum through fibrin membranes. The characteristic reduction in the total proteins with a disproportionate reduction in the concentration of the larger particles, the globulins, and, in consequence, a reversal of the albumin-globulin ratio can be readily shown at all pressures. The concentration of antibodies around inflammatory lesions, demonstrated by Fox (1936), could be also similarly explained.

This conception of a selective barrier between the lesion and the circulation is clearly important in relation to the ideas current on the nature of toxaemia, and on vaccine therapy. While readily diffusing toxins, such as that of diphtheria, may pass into the tissues through this barrier, most bacterial products will not. The theory of "toxaemia" based on the existence of toxic substances of the size of polypeptides, or other breakdown products of protein, is supported.

The treatment of a focus of sepsis by a vaccine may be removed from the realm of the empirical into which it had drifted, in that the focus itself will have little immunizing value in the absence of a septicaemic spread.

The size of the actual pores in fibrin has unfortunately not been established.

The separation of the vital dyes, vital new red and trypan blue can be obtained with gradocol collodion membranes of 0·003 mm. average pore size, under the same conditions as those used for the fibrin membranes, but, at that porosity, no proteins will pass the collodion. It must be concluded that fibrin and collodion do not behave in exactly the same way towards the range of substances I am using, and it is not possible to argue from one to the other. We shall be obliged to await the verdict of the physicist on this point.

Since concluding this work a preliminary note has appeared (Edlund, 1948) on the absorption of proteins and bacteria from normal and infected joints in normal and heparinized animals. It would appear that the results obtained, when available, will be very similar to those given here.

SUMMARY.

Membranes prepared from human fibrin were used as filters to determine whether a fibrin barrier would explain—

(*a*) The exclusion of trypan blue and similar dyes from areas of inflammation.

(*b*) The failure of some localized foci to stimulate immunity.

(*c*) The composition of inflammatory exudates.

It was found that, while trypan blue and some other vital dyes were retained, vital new red passed through. Anything as large as a micro-organism is held back, but the bacterial toxins can be forced through in filtration under considerably reduced pressure. They do not readily pass at low pressures or by diffusion.

Leucocytes can pass these membranes.

When serum is filtered or diffuses through fibrin the globulin is concentrated behind the filter, the filtrate has the composition of an inflammatory exudate with a lower total protein and reversal of the albumin-globulin ratio. Specific antibodies, being globulins, act in the same way.

I have received much help from my colleagues at St. Mary's, my thanks being due particularly to Mr. L. B. Holt for supplies of toxins, and for carrying out independent estimations on many of the filtrates from them; to Dr. F. Himmelweit for his advice on filters and filtration; and to Dr. S. Jacobs for technical assistance and for making available to me his manuscripts and thesis on filters.

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VIRULENCE AND TOXIGENICITY OF DIFFERENT SEROLOGICAL TYPES OF *C. DIPHTHERIAE*.

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THE Gravis, Mitis and Intermedius Groups of *C. diphtheriae* have each been subdivided into a number of distinct serological types (Hewitt, 1947*a*), and the correlation of other biological properties with the serological type is being attempted. Some observations on the haemolytic activity of different strains have already been reported (Hewitt, 1947*b*) and attention is now directed to their virulence and toxigenicity.

A clear distinction has been made (Roux and Yersin, 1889) between virulence, or the lethal power of living bacteria, and toxigenicity, or the power of producing soluble toxin, but it is not clear whether these functions are related or whether they vary independently. It is possible that virulent bacteria might fail to produce soluble toxin *in vitro*, and on the other hand strains producing toxin in culture filtrates might fail to kill animals when living cultures were injected. There is a further possibility that strains of different serological types might produce toxins which differ and are not neutralized by the same antitoxin.

Another matter to be determined is whether virulence and toxigenicity are constant properties of any given serological type, or whether they vary among different strains of the same serological type.

In view of the varying senses in which they are used it is necessary to define the terms virulence and toxigenicity as they are used in this communication. By virulence is meant the lethal power of living bacteria when injected subcutaneously into guinea-pigs, and by toxigenicity is meant the power of producing soluble toxin when the bacteria are cultured in a medium known to yield potent toxins with a number of common strains.

MATERIAL AND METHODS.

Strains.

Both recently isolated strains from this country and abroad and older laboratory cultures were investigated. Methods of isolation, culture, group differentiation and serological typing have been described previously (Hewitt, 1947*a*, *b*).

Virulence testing.

Since one of the objects of this investigation was to detect any lack of correlation between virulence and toxin production, it was decided not to use the test for virulence which depends upon the skin reaction, but to use the alternative method which depends upon the lethal effect of the culture on guinea-pigs.

Strains were sub-cultured on small Loeffler slopes in 5 c.c. screw-capped

bottles with a culture surface of a little over 2 sq. cm., and after 18 hours' incubation the bacteria were examined for purity and suspended in 2 c.c. of broth. 0·5 c.c. of the bacterial suspension was injected subcutaneously into each of two guinea-pigs weighing between 250 and 350 g., one of which had received 500 units of diphtheria antitoxin intraperitoneally 18 hours previously. The experimental animals were kept under observation and weighed daily for ten days if death had not occurred previously.

Deaths from diphtheritic toxaemia occurred sometimes within 24 hours (26 per cent of the deaths) and usually within 48 hours (96 per cent), but a few (4 per cent) died on the third day. With two strains when the inoculum was small owing to poor growth, death occurred after six and eight days respectively.

In all 212 strains were tested for virulence; of these 170 were virulent and 42 failed to kill the injected animal. In every case the guinea-pig injected previously with diphtheria antitoxin survived so that no strain was found of which the living culture killed the animals other than by the production of diphtheria toxin.

Toxigenicity.

After previous subculture on Loeffler slopes strains were inoculated into broth in 500 c.c. quantities contained in 160-oz. bottles. The broth used was prepared by digestion of horse flesh with papain for two and a half hours, fermentable carbohydrates were removed by yeast, acetic acid (0·5 per cent) sodium lactate (0·1 per cent) and maltose were added and the medium was sterilized by filtration. The final broth contained about 0·3 per cent of total nitrogen and 0·1 per cent of amino-nitrogen, and yielded potent toxins when inoculated with Park-Williams strain and Gravis type I, II and III strains.

Fairly large batches of medium were prepared and used for a series of experiments. In each experiment a control was included to ensure constant conditions, the control being a strain of established toxigenic properties.

Bottles of broth were incubated at 36° for ten days for toxin production, the air supply of the culture being increased by incubating the bottles on their sides instead of upright. At the end of incubation toluene was added, and after standing at room temperature overnight the main pellicle of growth was removed by filtration through folded filter-paper and the clear filtrates were filtered through Ford filter pads in order to render them sterile. The potency of the toxins was determined by an intracutaneous method in guinea-pigs (Römer, 1909).

Serial dilutions of the toxin to be tested were mixed with an equal volume of either 0·002 or 0·02 units of diphtheria antitoxin and 0·2 c.c. of the mixture was injected intracutaneously. The erythematous skin reaction was then read after 48 hours. Toxins giving no reaction when mixed with antitoxin were injected alone to confirm the absence of toxin. Results were calculated in Lr doses, that is the amount of toxin that will give a standard skin reaction when mixed with 1 unit of diphtheria antitoxin, and in the tables results are given in terms of Lr units, that is the number of Lr doses per ml. Lr units and flocculating (Lf) units are approximately equivalent. With the more potent toxins results were confirmed by flocculation tests, but this was not possible in the case of weaker toxins.

Altogether over 600 culture filtrates were prepared and the potencies ranged

from 0·002 to 75 units of diphtheria toxin per ml., the high values being obtained from filtrates of a laboratory culture of the original toxigenic Park-Williams 8 strain. Among other strains the most potent varied from 6 to 13 units per ml. Thirty-six filtrates contained no toxin at all, and gave no skin reaction even when injected alone without diphtheria antitoxin, whereas a potent toxin will give skin reactions in dilutions of 1 in 100,000 or more.

Further investigations on this point are planned, but so far no qualitative differences between toxins from strains of different types have been observed.

Correlation of Virulence and Toxigenicity.

In the case of 208 of the strains both the lethal effect on subcutaneous injection of the bacteria and the toxin content of sterile culture filtrates measured by skin test were determined. 170 strains were virulent and toxigenic and 38 strains were avirulent and nontoxigenic. In every case the virulence and toxigenicity tests agreed, so that it is confirmed that the lethal effect of whole cultures is due to toxin production.

Although, broadly speaking, strains producing most toxin *in vitro* killed guinea-pigs most quickly when the bacteria were injected subcutaneously, the correlation was not perfect, a probable reason for this being that the culture conditions necessary for maximal toxin production vary from strain to strain and cultural conditions *in vivo* and *in vitro* are very different.

TABLE I.—*Speed of Killing and Toxigenicity.*

		Number of animals dying after—			
		0–24 hrs. 45	24–48 hrs. 118	48–72 hrs. 7	Survived. 42
In vitro toxin production (Lr units)	*Lowest*	0·1	0·05	0·03	0
	Highest	13·0	10·0	1·5	0
	Mean	2·6	1·85	0·6	0

Note.—The laboratory culture strain Park-Williams 8 is omitted from various tables, since its toxigenicity has been enhanced selectively in laboratory sub-cultures for many years, and its abnormally high toxigenicity obscures the statistical examination of the results with recently isolated strains.

Toxin Production in vivo.

In laboratory culture media maximal toxin production does not usually occur for several days and may take over a week, but when injected into guinea-pigs cultures kill the animal within two days, and this period includes the production of the toxin as well as the time for the toxin to produce its effect on the body. This is especially remarkable in the case of strains which kill the animal in 24 hours.

An experiment was performed with eight rapidly lethal strains in order to test whether early deaths were due to the injection of preformed toxin. The 18 hours' growths on Loeffler slopes were suspended in broth (approximately 1 sq. cm. of growth in 1 ml. of broth), and a 0·5 ml. sample of each was injected into a separate guinea-pig. Seven of the eight animals were dead in less than 30 hours, and the eighth died in less than 44 hours. Another sample of each suspension was centrifuged for 15 minutes at 1500 r.p.m., and the bacteria which

had been sedimented were suspended in broth of the same volume as the supernatant fluid. Guinea-pigs were injected subcutaneously with 0·5 ml. of the re-suspended bacteria. Five were dead within 30 hours, and the remaining three animals died in less than 44 hours. The supernatant fluids from the centrifuge tubes which still contained some bacteria were also injected subcutaneously in 0·5 ml. doses. One of the animals died in 25 hours, six more died in less than 44 hours, and the remaining animal died in 50 hours. A further sample of each of the original suspended cultures (9 ml.) was filtered through a sterile 3 cm. Seitz filter and 0·5 ml. of each of these filtrates was injected subcutaneously. None of these guinea-pigs died.

This experiment indicates that the lethal effect of the cultures was due to toxin formed after injection into the guinea-pigs, and not to preformed toxin produced during *in vitro* incubation of the culture. There was a step-wise decrease in quick killing power as the number of viable organisms fell in the series of experiments from the original untreated suspension, the centrifuged and re-suspended suspension, the centrifuged supernatant fluid and the sterile filtrate. The centrifuge fractions were included in the test in order to eliminate the possibility that the lethal effects of whole unwashed cultures were due to preformed toxin, but that the toxin might be lost during Seitz filtration.

Gravis, Intermedius and Mitis Groups.

The virulence of the different groups is summarized in Table II.

TABLE II.—*Virulence of Gravis, Intermedius and Mitis Strains.*

	Number of strains tested.	Virulent.	Avirulent.
Total	274	232	42
Gravis	71	65	6
Intermedius	23	23	0
Mitis	180	144	36

It will be seen that 8 per cent of the Gravis and 20 per cent of the Mitis strains were avirulent, but none of the Intermedius group.

TABLE III.—*Time of Death of Different Groups.*

	Number of animals dying after—			
	0–24 hrs.	24–48 hrs.	48–72 hrs.	Survived.
Gravis	7	45	4	6
Intermedius	3	12	0	0
Mitis	35	61	3	36

As would be expected from the more heterogeneous nature of the Mitis group there is a greater spread of the results than with the Intermedius or Gravis groups, but there is no evidence that the Gravis strains are more rapidly lethal than Mitis strains (Table III).

It should be noted that two saccharose fermenting strains of Mitis Type XXXII have been encountered which were both virulent and toxigenic. Saccharose fermenting diphtheria-like organisms are occasionally encountered in the clinical

laboratory, and these should not be discarded with a negative diphtheria diagnosis without a virulence test.

The results of toxigenicity experiments are given in Table IV. Avirulent strains and the laboratory Park-Williams 8 strain are excluded from the table.

TABLE IV.—*Toxin Production by Different Groups* (*Results are given in Lr Units*).

Group.	Number of strains.	Range.	Mean toxin content of filtrate.
Gravis	63	0·02 to 5	1·0
Intermedius	17	0·1 „ 6	1·4
Mitis	127	0·02 „ 13	2·7

Some Intermedius strains even after laboratory sub-cultures grow feebly in broth and some of these produce only 0·002 units of toxin, but these are not included in the table.

In laboratory culture in broth the Mitis strains, on the average, produced most toxin and the Gravis strains least, but by examining different sample strains it would, of course, be possible to weight the results in any given direction.

Gravis strains.

Toxin production of the different serological types of Gravis strains is summarized in Table V. It should be noted that in the case of some serological types only one or two strains have so far been isolated.

CORRELATION WITH SEROLOGICAL TYPE.

Gravis strains.

TABLE V.—*Virulence and Toxigenicity of Different Gravis Types.* (*Results in Lr Units*).

Serological type.	Haemolytic activity.	Number of strains.		Toxin production.		
		Virulent.	Avirulent.	Lowest.	Highest.	Mean.
G I	+ or 0	35	0	0·2	6·0	1·4
G II	+	25	0	0·1	5·0	1·4
G III	0	4	0	0·2	3·0	0·6
G IV	0	7	0	0·05	0·5	0·3
G V	0	2	0	0·3	1·0	0·7
G VI	0	4	0	0·1	2·0	0·7
G VII	+	1	0	1·5	..	1·5
G VIII	+	0	1	0	..	0
G IX	0	3	0	0·02	0·2	0·12
G X	+	3	0	1·0	1·5	1·2
G XI	0	1	0	3·0	..	3·0
G XII	0	1	0	0·6	..	0·6
G XIII	+	1	0	0·6	..	0·6
Doubtful	0	0	5	0	..	0
Total		87	6	0·02	5·0	1·0

TABLE VI.—*Toxigenicity of Mitis Serological Types (Toxin Potencies in Lr Units).*

Serological type.	Haemolytic activity.	Number of strains.		Toxin production.		
		Virulent.	Avirulent.	Lowest.	Highest.	Mean.
M I	+	24	0	0·6	7·0	3·1
M II	0	10	0	0·1	2·0	0·8
M III*a*	+	12	0	0·3	7·0	2·8
M III*b*	0	0	15	0	0	0
M IV	+	14	0	0·7	4·0	1·6
M V*a*	+	8	0	0·1	3·5	1·4
M V*b*	+	0	3	0	0	0
M VI	+	7	0	0·5	4·0	1·6
M VII	+	10	0	0·5	5·0	3·2
M VIII	+	6	0	0·5	4·0	2·0
M IX	+	3	0	1·8	4·0	2·9
M X	+	6	0	1·0	3·0	1·9
M XI	+	5	0	1·5	5·0	3·5
M XII	+	4	0	0·02	2·5	1·3
M XIII	+	5	0	1·2	3·5	2·3
M XIV	+	1	0	75·0	..	75·0
M XV	+	1	0	2·0	..	2·0
M XVI	0	1	0	0·6	..	0·6
M XVII	+	1	0	1·3	..	1·3
M XVIII	+	2	0	1·3	10·0	5·7
M XIX	+	1	0	0·5	..	0·5
M XX	+	1	0	3·0	..	3·0
M XXI	+	1	0	1·5	..	1·5
M XXII	+	6	0	1·0	6·0	3·0
M XXIII	+	0	1	0	..	0
M XXIV	+	1	0	0·5	..	0·5
M XXV	+	2	0	1·5	2·0	1·8
M XXVI	+	1	0	1·5	..	1·5
M XXVII	+	6	0	1·0	4·0	2·0
M XXVIII	+	2	0	2·0	6·0	4·0
M XXIX	+	6	0	1·0	6·0	3·0
M XXX	0	0	2	0	0	0
M XXXI	+	0	3	0	0	0
M XXXII	+	2	0	0·5	1·7	1·1
M XXXIII	0	0	1	0	..	0
M XXXIV	0	1	0	4·0	..	4·0
M XXXV	+	2	0	3·0	10·0	6·5
Doubtful	..	14	11	1·3	13·0	4·3
Total		156	36	0·02	13·0	2·7

Note.—In calculating mean toxin values avirulent strains and Park-Williams 8 strain are omitted.

Twelve of the thirteen serological types contained only toxigenic strains, but six of the seven non-toxigenic strains were of doubtful type, some of which showed signs of being serologically related to Types III and IV.

The clinical interpretation of infections with non-toxigenic diphtheria-like organisms is difficult. Clinically such infections are occasionally reported to be of moderate severity, although toxaemic symptoms are absent.

Mitis strains.

The toxigenicities of strains belonging to 35 mitis types are summarized in Table VI.

Some interesting points arise from Table VI. On the whole each serological group appears to be uniform in consisting of all toxigenic or all non-toxigenic strains, although the numbers in the case of some types are too small for final conclusions: 28 types consist of entirely toxigenic strains and 4 types are non-toxigenic, but Types III and V can be subdivided quite sharply into toxigenic and non-toxigenic strains. In the case of Type III the toxigenic and virulent strains are haemolytic, whilst the non-toxigenic avirulent strains are non-haemolytic. It would appear that the non-haemolytic subtype strains of Type III are capable of retaining their antigenic structure whilst simultaneously losing both their virulence and haemolytic activity.

Type II should also be noted since this Mitis type is serologically indistinguishable from Gravis Type II. Both Mitis and Gravis Type II are virulent, but whilst Gravis Type II is haemolytic, all the strains of Mitis Type II encountered have been non-haemolytic. Thirty-one Mitis strains were encountered which were non-haemolytic; 26 of these belong to Types II or IIIB, whilst 160 strains were haemolytic.

Selection of colonies.

Using the selenite medium described previously (Hewitt, 1947*a*) it is possible to pick colonies differing considerably in appearance and size, and the toxigenicities of some of these variants were examined.

TABLE VII.—*Toxigenicity of Colonial Variants* (*Results in Lr Units per ml.*).

	Original culture.	Variants. I.	Variants. II.
Gravis IV	..	0·05	0·04
Mitis II	0·5	0·25	0·12
„ XXI	1·5	0·7	1·5
„ XXV	1·5	2·0	1·5

DISCUSSION.

Although it is generally thought that the lethal effect of cultures of *C. diphtheriae* is due to the soluble exo-toxin, it is probable that other factors are of importance in the disease besides the toxin. In the present experiments, however, there was a close connection between the killing power of the bacteria when injected into guinea-pigs and the ability of the organisms to produce skin-reacting toxin in broth cultures. The lethal power of *C. diphtheriae* in guinea-pigs appeared

to be entirely toxaemic, so that it might be more informative to speak of toxigenic strains rather than virulent, and to reserve the term virulent to describe the bacteria which are able to multiply in animals from very small inocula. In general a massive dose of *C. diphtheriae* is required to kill an animal.

Unlike such organisms as the haemolytic streptococcus, it is extremely difficult to determine with any accuracy the number of *C. diphtheriae* required to kill an animal, so that the only quantitative figure one can readily obtain is that of *in vitro* toxigenicity, or the amount of toxin produced in a standard culture medium. The toxigenicity of over 300 strains was determined, some of them on several occasions. Although more Mitis strains (28 per cent) were avirulent and non-toxigenic than Gravis strains (12 per cent), the average value of the toxin produced on the culture medium used by toxigenic Gravis strains (1·0 Lr units per ml.) was not greater than that of Mitis strains (2·7 units). All Intermedius strains examined were toxigenic, and the average toxigenicity (1·4 units) was intermediate between that of Mitis and Gravis strains. The wide variation in toxigenicity of Mitis strains accords with the heterogeneity of the group discussed in a former communication (Hewitt, 1947*a*).

It would be of interest to find a laboratory test that would account for the greater clinical severity of the disease caused by some strains of *C. diphtheriae*, e.g. some Gravis strains, but in the present experiments the *in vitro* toxigenicity of Gravis strains was not greater than that of Mitis strains, and further search for an explanation must be sought.

The evidence accumulated from experiments on several hundred strains suggests that both lethal power (virulence) and toxigenicity can be correlated with serological type, although the possibility cannot be excluded that by multiplication of the number of strains examined exceptions might be found.

In the Gravis group all the serological types are virulent with the exception of two, and it is possible that these atypical strains should be excluded from the Gravis group and regarded as atypical avirulent starch-fermenting Mitis types. The question arises as to whether an organism that does not produce diphtheria toxin should be described as *C. diphtheriae* at all, but the serological examination of the Mitis group lends support to the view that all members of the species do not produce toxin. In at least two serological types of the Mitis group both virulent and avirulent members are found which are indistinguishable serologically. In the case of Type III the virulent strains are haemolytic, whilst the avirulent strains are not. This correlation of virulence with haemolytic activity is not general, however, since virulent Gravis Type I organisms are isolated, both haemolytic and non-haemolytic. Also Gravis Type II and Mitis Type II organisms are indistinguishable serologically, but differ in haemolytic activity, the Gravis group being haemolytic and the Mitis non-haemolytic, but both Gravis and Mitis are virulent.

In a former communication the evolution of *C. diphtheriae* was commented upon, the possibility being envisaged of Mitis strains being variants of Gravis strains which had lost their starch-fermenting ability, and the Mitis strains then undergoing variation in antigenic structure. In the case of strains of a single serological Mitis type varying in virulence, it would appear that a further stage of degradation is reached when the strain loses its virulence and toxigenicity. The occurrence of completely avirulent strains of *C. diphtheriae* makes formidable the task of distinguishing between the true diphtheria bacilli and some diph-

theroids, since they are frequently similar in colonial form, morphology and fermentation reactions; serological typing appears the only method of final classification. A further difficulty in diagnosis is Mitis Type XXXII, of which the strains so far encountered ferment saccharose and are virulent and toxigenic.

In a recent paper McLeod and Robinson (1948) mention avirulent diphtheria strains, some of which are described as Gravis and others as atypical.

So far no evidence has been obtained of any qualitative differences between the toxins produced by different strains, and in every case ordinary diphtheria antitoxin purified by enzyme digestion protected animals against living cultures of every serological type.

SUMMARY.

1. Strains of *C. diphtheriae* which proved lethal when living bacteria were injected into guinea-pigs all produced soluble toxin in laboratory culture media and avirulent strains were non-toxigenic. This correlation applied to all serological types examined.

2. Diphtheria antitoxin neutralizes the toxins, and protects guinea-pigs against the lethal effects of living cultures of all serological types.

3. More Mitis than Gravis strains were avirulent and non-toxigenic, but the average *in vitro* toxigenicity of Mitis strains tended to be higher than that of Gravis strains.

4. There is a correlation between serological type and toxigenicity, but some Mitis types could be sharply subdivided into virulent and avirulent sub-types.

5. Mitis Type II and Gravis Type II strains are indistinguishable serologically, both classes are virulent and toxigenic, but they differ in starch fermentation and haemolytic activity.

6. Virulence and haemolytic activity occur independently in different strains.

7. The occurrence of non-virulent strains which are otherwise typical *C. diphtheriae* and of virulent, toxigenic strains which ferment saccharose raises difficulties in the diagnosis of infections.

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